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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTOR(S): David I. Cohen

TITLE: IMMUNOMODULATORY CHEMICAL ENTITIES AND RELATED METHODS FOR THEIR DISCOVERY AND USE

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UNITED STATES PROVISIONAL PATENT APPLICATION

DAVID I. COHEN

FILED MARCH 16, 2004

IMMUNOMODULATORY CHEMICAL ENTITIES AND RELATED METHODS FOR THEIR DISCOVERY AND USE

BACKGROUND OF THE INVENTION

Cancers and chronic infections are the most prominent examples of common human diseases that respond to immune-based treatments. Although infections were the first diseases to be controlled by immunization, a series of clinical trials in humans starting in the 1980s have established that an immune response, particularly of the cytotoxic T lymphocyte (CTL) arm of the immune system, could regress some human melanoma (Phan, C.Q., et al. (2003), "Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma," Proc. Natl. Acad. Sci. USA 100:8372-7) and renal cancers. These observations were broadened by the discovery that dendritic cells (DC), a specific class of antigen-presenting cells (APC), are particularly effective at initiating CTL activity against cancers and other diseases (Banchereau, J. et al. (2001), "Dendritic cells as vectors for therapy," Cell 106:271-4; Dalyot-Herman, N. et al. (2000), "Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed APC," J. Immunol. 165:6731-7). Technologies that target and activate DC have yielded some early successes against human cervical pre-malignancies, caused by infection with Human Papilloma Virus (HPV) and human lung cancer. In contrast to chemotherapeutic drugs currently used against cancer, agents that provoke a CTL

response against cancer potentially are accompanied by few side effects, owing to the great specificity of the immune response.

Efforts to develop immunotherapeutic drugs that treat cancer have been hampered by technical difficulties in targeting and activating DC to deliver and sustain the required entry signals to the CTL. Antigen targeting for the induction of a CTL response is a challenge insofar as natural processing requires that the antigen enter the cytoplasm of the cell in order to bind to the immune system's major histocompatibility complex (MHC) Class I antigen, a prerequisite to CTL activation because the ligand for activating the T cell receptor on CTL is a complex of antigen and MHC Class I. In almost all cases protein antigens, even when they are coupled with a DC co-activator, enter exclusively into the alternative MHC Class II antigen presentation pathway that excludes CTL stimulation. This can be overcome in part by peptide-based technologies, because peptides bind to MHC Class I that is already on the surface of the DC. However, this technology is non-specific and most peptides are poor DC activators which limits their efficacy as human treatments for cancer.

A limited group of biological proteins are known to stimulate a CTL response ("base compounds"). Although the mechanism by which these base compounds work has not been previously established, this filing discloses biologics (PrecisionConjugates™, trademark owned by the inventor, the cancer vaccine of the present invention) that can stimulate a CTL response via the DC. The present inventor has unexpectedly determined that variants and derivatives of the Human Immunodeficiency Virus 1 (HIV-1) trans-activator of transcription (Tat) can stimulate this CTL response (Moy, P. et al. (1996), "Tat-mediated protein delivery can facilitate MHC

class I presentation of antigens," Mol. Biotechnol. 6:105-13; Fanales-Belasio, E. et al. (2002), "Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses," J. Immunol. 168:197-206). Native Tat is an immunosuppressant Viscidi, R.P. et al (1989), "Inhibition of antigen-induced lymphocyte proliferation by Tat protein fromHIV-1," Science 146:1606-8). Additional biologics that are currently known to directly trigger a CTL response are based on Heat Shock Proteins (HSP) (Suzue, K. et al. (1997), "Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway," Immunology 94:13146-51; Stebbing, J. et al. (2003), "Disease-associated dendritic cells respond to disease-specific antigens through the common heal shock protein receptor," Blood 102:1808-14), or on the outer coat protein of certain bacteria. HSP are showing limited efficacy in the treatment of certain genital neoplasms related to HPV infection. The CTL responses elicited by the cancer vaccine of the present invention are superior in animal models to HSP.

The present inventor was the first to reveal that the HIV-1 Tat molecule worked by specifically targeting and activating precursor APC. It is the non-binding hypothesis of the present inventor that base compounds work in substantially the same fashion. It is an additional non-binding hypothesis of the present inventor that Tat activates APC through a receptor-mediated mechanism. Tat motifs (DNA structures translated into protein) are revealed that have the structure of a receptor ligand. This discovery predicts derivatizations in the Tat molecule that could improve its ability to activate DC and methods to design novel adjuvants that could substitute for the DC-activating principle in Tat.

The present inventor's initial discovery of the APC targeting and activation properties of Tat resulted from work on suppression originating from the Tat molecule (Cohen, S.S. et al. (1999), "Pronounced acute immunosuppression in vivo mediated by HIV-1 Tat challenge," Proc. Natl. Acad. Sci. USA 96:10842-47). Tat is a small protein (14 kd on reducing gels) synthesized from the genetic sequence of the HIV-1, but it is not a structural protein that is contained in the HIV-1 virion itself. Instead, a large body of literature supports the proposal that Tat can be secreted from an infected cell and taken up by an uninfected cell, where its uptake results in transactivation of transcripts, a subset of which stimulate the cell (Frankel, A.D. and Pabo, C.O. (1988), "Cellular uptake of the Tat protein from Human Immunodeficiency Virus," Cell 55:1189-93) and a subset of which initiate programmed cell death. These observations demonstrate that Tat enters the cytoplasm of cells, where transactivation is mediated, but they did not establish the key mechanism of entry via the receptor. Additional research confirmed by the present inventor showed that Tat initiated a suppression that was antigen-specific (PrecisionTolerogens™, (PT) trademark owned by the inventor, the tolerogen of the present invention). In this property tolerogens of the present invention are distinct from immunosuppressants currently used in human therapy.

Biological agents currently used to treat disease introduce foreign protein (monoclonal antibodies, insulin, Factor VIII, organ transplants) into the body. An immune response against these biologicals is undesirable because this immunity neutralizes, or in the case of organ transplants, rejects the biological in addition to causing collateral damage through allergic and autoimmune reactions. Recombinant proteins of human origin have been very successful in sustaining the efficacy of

biological therapies in many cases, such as insulin, Factor VIII, and "humanized" monoclonal antibodies. Even in these successes undesired autoantibodies accumulate over time that limit or terminate efficacy. Methods to ameliorate these undesirable immune responses have not yet been developed.

Autoimmune diseases are a series of unwanted immune responses that selectively destroy tissues. Severe autoimmune diseases are chronic, debilitating, and life-threatening. In some cases, specific agents that provoke a particular type of autoimmune disease are becoming defined. Approximately 2.5 million individuals currently suffer from rheumatoid arthritis (RA) in the US alone. Severe RA accelerates death rates at least five-fold compared to the general population (Wolfe, F. et al. (2003), "Predicting mortality in patients with RA," Arth. Rheumatism 48:1530-42). Peptide fragments from collagen type II, an important structural component in undamaged joints, can provoke RA in animals and could be developed as vaccines against human RA (Van den Steen, P. et al. (2002), "Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis," FASEB J. 16:379-89). Such collagen type II peptides are one important example of fragments that could be fitted in to PT rendering them into specific therapeutics for autoimmune disease, in this preferred example for RA.

The benefit of the tolerogen of the present invention is its potential to avoid the immunocompromised state induced by current standards of immunosuppressive therapy. This benefit is particularly important in situations where chronic treatment is needed, such as occurs following organ transplantation or during autoimmune disease.

However, these benefits require a high specific activity tolerogen, which can be produced as a result of structural resolutions in the Tat protein that are revealed in this filing. In the absence of structural resolutions and a thorough understanding of the mechanism of Tat suppression, both revealed here, it has not been possible to rationally design and test tolerogens that maintain the specificity and activity of Tat in living organisms.

The constructs and designs taught here allow for the stable production of active Tat. The stability of the design is a critical feature allowing the tolerogen of the present invention to be used in organ transplantation. The nature of this embodiment of the invention allows the insertion of any specific antigen into a tolerogen "cassette" described herein, in which tolerance will result to that antigen exclusively while the remainder of the immune system remains intact. A particularly beneficial tolerogen cassette would include the immunoglobulin VH and/or VL region from any human or murine monoclonal antibody (Mab), particularly a Mab directed against a cancer growth antigen. In this process the tolerogen of the present invention converts experimental tools in the mouse into potential drugs for human cancer whose efficacy can be established through clinical trials. The tolerogen of the present invention would be administered prior to and/or along with the immunogenic biological in order to ensure an antigen-specific tolerized state in the patient.

In order to make the cancer vaccine of the present invention for the treatment of cancer, it is necessary to remove, modify, or override through mutation the suppressive principle in Tat such that DC activation is maintained. Again based upon structural resolutions, the present inventor describes a critical SH3-binding motif within the Tat

sequence that controls the generation of a highly immunosuppressive antigen presenting cell regulatory macrophages (AReg). Simian lentiviruses related to HIV-1 but not causing immunodeficiency have an alternative motif that is not suppressive. We teach here that the mutant protein in a mouse strain (*hairless, hr)* that develops an immunodeficiency strikingly parallel to that seen in HIV-1 infection, including lost CTL and poor APC functions, encodes a homologous SH3 binding motif to HIV-1 Tat. This SH3 motif is proposed to control the differentiation potential of monocyte precursors either into DC that stimulate CTL, or AReg that suppress CTL. While prominent in HIV-1 infection, AReg are also now discovered in the literature as critical contributors to invasion of gastric (Ishigami, S. et al. (2003), "Tumor-associated macrophage (TAM) infiltration in gastric cancer," Anticancer Res. 23:4079-83), pancreas (von Bernstorff, W. et al. (2001), "Systemic and local immunosuppression in pancreatic cancer patients," Clin. Cancer Res. 7:925s-32s), and ductal infiltrating breast tumors (Lin, E.Y. et al. (2002), "The macrophage growth factor CSF-1 in mammary gland development and tumor progression," J. Mammary Gland Biol. Neoplasia 7:147-62; Visscher, D.W. et al. (1995), "Clinicopathologic analysis of macrophage infiltrates in breast carcinoma," Pathol. Res. Pract. 191:1133-9), as well as components of tolerance in organ transplantation.

In one of its most exciting applications, this filing teaches how to develop New Immunomodulatory Chemical Entities (NICE). It is the non-binding theory of the present inventor that initial screening of NICE is a critical step that has plagued the development of rationally-designed drugs by the pharmaceutical industry as unexpected patterns of activity have emerged at advanced stages of testing. Thus the original promise of being

able to control undesirable cellular processes such as cancer and autoimmunity through small molecule intervention at discrete SH3 and SH2 domains (Koch, C.A. et al. (1991), "SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins," Science 252:668-74 has not yet been realized despite intense investment of time and resources by major pharmaceuticals because of lack of specificity of the NICE.

This filing teaches how to derivatize, swap, or mutate the SH3 binding motif of Tat to use as a screening tool to increase the specificity of small molecules that maintain or enhance DC function (immunostimulatory NICE) or that enhance AReg function (tolerogenic NICE). In this process, a series of closely related structures are created from natural variants and random mutations that will be used to differentially screen for NICE active at this motif. This mutational library will allow the exact identification of high specific-activity NICE with high affinity for actively suppressive motifs (or dominant negative mutations competing for the same site) but not related, inactive, mutations with alternative binding specificities. The present inventor has therefore designed a series of "filters" to identify high specific activity molecules and "filter out" molecules with poor specificity from the screening compound libraries. Both positive filters (suppressive motifs and related dominant negatives) and negative filters (inactive mutants and natural variants) are employed to isolate NICE with the most exact binding characteristics.

NICE scoring positive through the entire series of filters can next be conveniently revalidated by an *in vitro* functional biological assay (DC index) developed by the present inventor for the purpose of developing NICE, thereby insuring that drug candidates screened at the plate have the expected activities on cells without causing

unsafe toxicity. The therapeutic action of immunostimulatory NICE can next be proven in vivo in the mouse *hr* model, the existence of a viable mouse model further insuring the practicality that specific drugs identified against this motif will be beneficial. At this point, and only after specificity and safety are thoroughly established both *in vitro* and *in vivo*, immunostimulatory NICE can be definitively tested in human diseases including cancer and chronic autoimmune and infectious diseases.

The successful development of NICE from this strategy will allow the retrograde establishment of optimal conditions for measuring and enhancing affinity while reducing non-specificity. Common variables that have not yet been standardized in the industry include buffering conditions, multimerization of the screening domains, and structuring. It may be learned, as examples of many possibilities, that tetramerized binding motifs (as proved useful in categorizing CTL receptors), that motifs embedded in stable structures such as the Tat motif which is flanked by proline residues, that alternative buffering conditions, that off as opposed to on rates, or that unexpected affinities are most accurate at predicting high specific activity interactions. The validation of NICE screening conditions will serve to unlock the potential of rational drug design for SH3 pathways, which cannot be achieved without such a well-defined process (taught here) leading from the molecule to the patient.

SUMMARY OF THE INVENTION

For the purposes of clarification and to avoid any possible confusion, the trans-activating (Tat) of the present invention will be designated as either "Tat" for

conventional immunosuppressive Tat protein and "Tat*" for Tat that is genetically or chemically derivatized so that it is stimulatory.

The following additional terms are also employed:

New Immunomodulatory Chemical Entities, the NICE of the present invention, in their preferred embodiment are small synthetic molecules or peptides, drive differentiation of monocytes into immunosuppressive antigen presenting cell regulatory macrophages (AReg) for the purpose of inducing antigen-specific tolerance (tolerogenic NICE) or drive differentiation into dendritic cells stimulating antigen-specific CTL responses for the treatment of cancer (immunostimulatory NICE).

PrecisionTolerogens™ (PT, trademark owned by the inventor), the tolerogen of the present invention, is a specific molecular structure that combines a designated tolerogenic antigen to derivatized or native immunosuppressive Tat such that the specific molecular structure tolerizes in an antigen specific manner. The tolerogen of the present invention can be accomplished via either recombinant DNA or protein constructions, or by administering the tolerogen and the antigen in a formulation that favors uptake of both individual components by the same antigen-presenting cell (APC). Such formulations include but are not limited to, lipid vesicles, sustained release transdermal patches, or sublingual tablets. The tolerogen of the present invention can also be accomplished using tolerogenic NICE by mixing or conjugating NICE to the tolerizing antigen.

PrecisionConjugates™ (trademark owned by the inventor), the cancer vaccine of the present invention, is a specific molecular structure or mixture that combines a designated cancer antigen (Ag) to derivatized non-immunosuppressive Tat such that

the specific molecular structure triggers an antigen-specific cytotoxic T lymphocyte (CTL) response via the dendritic cell (DC). The cancer vaccine of the present invention can be accomplished via either recombinant DNA or protein constructions or a mixture of Tat* (or immunostimulatory NICE) and antigen. Additionally, immunostimulatory NICE can be used as an adjuvant and mixed with a selected cancer antigen.

The present invention provides cancer vaccines, adjuvants and immunotherapeutics for preventing and treating cancer, related immunotherapeutics for induction of tolerance to foreign antigens and a method for drug discovery of New Immunomodulatory Chemical Entities (NICE). The present inventions further provide methods for treating cancer and preventing undesirable immune responses to foreign antigens and autoimmune diseases with NICE. The present inventions further describe motifs and their DNA sequence that can be realized as siRNA for the purpose of interfering with AReg development, as a further method to treat cancer (or Human Immunodeficiency Virus Type 1 (HIV-1) infection), or that can interfere with replacement sequences favoring DC development, as a further method to induce antigen-specific tolerance.

The present inventor has unexpectedly demonstrated that Tat mediates two independent activities, a receptor-mediated triggering event at the cellular surface and an intracellular trans-activation that controls antigen-presenting cell (APC) differentiation. The receptor-mediated triggering is specific to APC, committing them for activation and differentiation, and mediated exclusively by multimeric Tat. The present invention identifies and defines a proline (P) rich stretch near the amino terminus of HIV-1 and HIV-2 Tat as a new SH3 binding domain. This transduction motif has

significant homology to the SH3-binding domain of the mouse gene hairless (*hr*) implying that the functions are related. Unexpectedly, mice with the *hr* mutation develop and AIDS-like syndrome characterized by poor CTL function, TH1 to TH2 shift, and increased susceptibility to chemical and ultraviolet light (UV)-induced skin cancers. Earlier evolutionary variants of Tat predominate in lentiviruses that infect monkey species that do not develop immunodeficiency and that do not have epidemic infection. These variant Tat do not have the SH3 binding domain and instead substitute a different sequence, also set off by P at either end of the sequence, into the transduction motif. Tat motifs (short DNA structures translated into protein) are revealed that have the structure of a receptor ligand. This invention describes derivatizations in the Tat molecule that could improve its ability to activate DC and methods to design novel adjuvants that could substitute for the DC-activating effects of derivatized Tat.

Additional methods to influence the SH3 control of dendritic cells involve activating RNA interference (RNAi), which results in sequence-specific degradation of the targeted double strand RNA (Fire, A. (1999), "RNA-triggered gene splicing," Trends Genet. 15:358-63; Zamore, P.D. (2001), "RNA interference: listening to the sound of silence," Nat. Struct. Biol. 8:746-50). RNAi is a therapeutic model of intense recent interest for specific suppression of RNA transcription that is broadly applicable to disease states such as cancerous cell growth and viral infections. Small interfering RNAs (siRNA) are RNA duplexes of 21-23 nucleotides that enter the RNAi pathway and silence the targeted transcript. An aspect of the current invention is to create RNAi interventions for cancer and certain infectious diseases, including HIV-1 infection, by using siRNAs to inhibit the AReg motif first identified from Tat. These siRNAs could be

directed against the SH3 binding motif of Tat or preferentially its cellular equivalent from *hairless* to reinvigorate an anti-cancer immune response. In HIV-1 infection, siRNAs suppressing the AReg motif in Tat would function both as immunostimulants and as inhibitors of the rate-limiting interface between HIV-1 growth and cellular transcription machinery. Although the parameters for siRNA efficacy are not well known, both the quality of the siRNA sequence (Ding, Y. and Lawrence, C.E. (2001), "Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond. Nucleic Acids Res. 29, 1034-46) and the capacity to deliver the siRNA into the cell (Brummelkamp, T.R. et al. (2002), "A system for stable expression of short interfering RNAs in mammalian cells," Science 296:550-3) are proposed to be essential determinants of efficacy. In the therapies of the present invention, that action occurs within DCs or ARegs, which are intensely phagocytotic cells, and can greatly facilitate achievement of therapeutic effects.

Another embodiment of the present invention is a method to stably produce biologically active Tat as a multimer. A further embodiment is a method to clone and characterize the Tat-binding receptor the APC. This characterization is useful to further enhance the targeting capabilities of Tat and to identify adjuvant compounds with APC triggering activities related to Tat. Genetic derivatives of Tat have been identified that, through modulating the signal transduction motif defined by the SH3 binding region, are predicted to differentiate predominantly dendritic cell (DC) responses or immunosuppressive antigen presenting cell regulatory macrophages (AReg). These motifs can be the targets of drug discovery aimed at influencing DC/AReg differentiation and thereby influence the immune response through NICE. The present invention

proposes a set of specific molecular structures based on Tat such that these structures either tolerize in an antigen-specific manner or trigger an antigen-specific CTL response via the dendritic cell.

BRIEF DESCRIPTION OF THE DRAWINGS

A detailed description of the invention is hereby described by non-limiting examples with specific reference being made to the drawings in which:

FIGS. **1**A-B depicts an analysis of Tat polymerization by protein immunoblot. (A) Control proteins (lane 1), HIV-1 Tat proteins (lanes 2,3), or recombinant Tat proteins (lane 4) were purified either from CD4+ T cell lines (lane 1), two cell lines transduced to express HIV-1 Tat proteins (lanes 2,3), or from *E. coli* transfected with (6xHis) tagged Tat by affinity chromatography to rabbit polyclonal anti-Tat antibodies (lanes 1-3) or His (lane 4). Eluted proteins were separated under non-denaturing conditions by SDS-PAGE electrophoresis. Proteins from paired gels were either transferred to nitrocellulose (A) and probed with a monoclonal anti-Tat antibody, or electroeluted after each lane was excised into two components (B), the first > 40 Mr (kDa) ("Upper") and the second <21.5 Mr (kDa) ("Lower"). Human monocytes were cultured in the presence of 50 ng/mL fractionated proteins for three days and the percent Fas Ligand expressing APCs represented activation.

FIG. **2** depicts fluorescence activated cell sorter analysis of the results of Tat activation of monocytes. Human peripheral blood monocytes were committed to differentiate into DCs through 5 days of culture in GM-CSF (100 ng/mL) and IL-4 (100 ng/mL). Committed DCs were cultured overnight either in medium alone (Control), LPS (100 ng/mL), or Tat (50nM), after which they were stained with an anti-CD86 antibody

and analyzed by FACScan for CD86, a specific marker of DC activation, induction (left panel) or generalized activation (right panel, enlargement into box R2, shown for Tat-stimulated cells).

FIG. **3** depicts the enhancement of antigen-specific activation of CTLs by Tat*-Ag complexes. Tat*, chemically derivatized so that it does not induce antigen presenting cell macrophage regulators (ARegs) from monocyte APC precursors (shown in FIG. **4** as oxTat) was biotinylated at its carboxyl terminus and conjugated to avidin-p24. Mice were immunized with Ag & Tat* (p24 in one flank and 5 μg Tat* in the other flank) or Ag alone in adjuvant on days 0 and 7. Control mice were given two injections of adjuvant. At day 14, draining lymph node cells from each animal were harvested and re-stimulated overnight in cultures of irradiated p24-transfected cells or control non-transfected cells. CTL activity was quantitated as the number of γ-interferon secreting spot forming colonies (SFC)/10^6 plated cells using ELISPOT assays.

FIG. **4** depicts median fluorescence of monocytes, cultured for six days either with no stimulus (0), 50 ng/mL TNFα, 100 ng/mL LPS, decreasing concentrations of C-Tat, or 50 nM oxidized ox-C-Tat and stained with an anti-FasL monoclonal antibody (Mab) followed by a fluorescinated (check sp.) goat anti-mouse polyclonal antibody.

FIG. **5** schematically depicts the means by which the Tat*-Ag conjugate cancer vaccine of the present invention activates cytotoxic T lymphocytes (CTL).

FIG. **6** depicts fluorescence-activated cell sorter analysis of mouse peritoneal macrophages that were isolated either after in vivo thioglycolate stimulation (Stimulated + adjuvant), or without in vivo stimulation (resting). Mouse peritoneal macrophages were cultured for five days wither in the absence of additional stimulation (C) with LPS

(100 ng/mL) or with Tat (100nM). Activation was determined as % enlarged cells (M1 fraction).

FIG. **7** depicts stable suppression of T lymphocytes by the Tat tolerogen of the present invention two weeks after immunization.

FIG. **8** depicts the antigen-specificity of Tat tolerogen of the present invention. Mice were immunized at day 0 and boosted at day 7 with an adjuvant emulsion containing either 5 µg Tat (Ag+Tol) biotinylated at its carboxyl terminus conjugated to avidin-p24, or with 5 µg avidin-p24 (Ag Alone) as control. At day 14, draining lymph node cells were harvested and stimulated with either specific or non-specific antigen and proliferation measured by ^{3}H thymidine uptake (CPM) after four days of culture.

FIG. **9** depicts fluorescence-activated cell sorter analysis of human peripheral blood monocytes cultured for four days in medium containing 5% fetal calf serum (Control), 50 nM Tat or 100 ng/mL LPS. Harvested cells were doubly stained with a fluoresceinated (anti-fl1) anti-Fas Ligand monoclonal antibody, and with an anti-CD14 rhodamine labeled Mab. Cells were analyzed by FACScan for activation (forward scatter), CD14 expression (% macrophages), and for induction of Fas Ligand (MFI). The T cell population is labeled R1.

FIG. **10**A-B depicts regulatory and immunosuppressive nature of Tat-activated macrophages. (A) Human polymorphonuclear neutrophils (PBMC) from one individual (PBMCs #3) cultured for 5 days in either medium with tetanus antigen (Ag, 0.3 Lf/mL), antigen with the further addition of 50 nM Tat (Ag+Tat) or Ag with 50 nM Tat and recombinant sFas protein (25 µg/mL) to block surface FasL expressed on macrophages (Ag+Tat+sFas). The results are graphed as stimulation index (mean cpm stimulated

culture/mean cpm medium control). (B) Proliferation of PBMCs cultured 6 days with either tetanus or *Candida* antigen alone (Ag), compared with cultures in which Tat (Ag+Tat, 125 nM), or Tat (125 nM) and the antagonistic anti-Fas antibody, ZB4 also were added (Ag+Tat+αFas).

FIG. **11** depicts the C-rich motif homologous to receptor binding ligands.

FIG. **12** depicts the membrane translocation domain (PTD) of Tat.

FIG. **13** depicts the SH3 binding domain within Tat.

FIG. **14**. schematically depicts the construction of cancer vaccine and tolerogen cassettes. Panel A: Domains of native Tat. Panel B: Varying antigen cassettes for the production of the cancer vaccines or tolerogens of the present invention. The immunostimulatory or immunosuppressive functions of domain 1 (SH3 binding motif) will determine if the resultant protein is a cancer vaccine (immunostimulant) or tolerogen (immunosuppressive).

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

For the purposes of clarification and to avoid any possible confusion, the trans-activating (Tat) of the present invention will be designated as either "Tat" for conventional immunosuppressive Tat protein and "Tat*" for Tat that is genetically or chemically derivatized so that it is stimulatory.

The following additional terms are also employed:

New Immunomodulatory Chemical Entities, the NICE of the present invention, in their preferred embodiment are small synthetic molecules or peptides, drive

differentiation of monocytes into immunosuppressive antigen presenting cell regulatory macrophages (AReg) for the purpose of inducing antigen-specific tolerance (tolerogenic NICE) or drive differentiation into dendritic cells stimulating antigen-specific CTL responses for the treatment of cancer (immunostimulatory NICE).

PrecisionTolerogens™ (PT, trademark owned by the inventor), the tolerogen of the present invention, is a specific molecular structure that combines a designated tolerogenic antigen to derivatized or native immunosuppressive Tat such that the specific molecular structure tolerizes in an antigen specific manner. The tolerogen of the present invention can be accomplished via either recombinant DNA or protein constructions, or by administering the tolerogen and the antigen in a formulation that favors uptake of both individual components by the same antigen-presenting cell (APC). Such formulations include but are not limited to, lipid vesicles, sustained release transdermal patches, or sublingual tablets. The tolerogen of the present invention can also be accomplished using tolerogenic NICE by mixing or conjugating NICE to the tolerizing antigen.

PrecisionConjugates™ (trademark owned by the inventor), the cancer vaccine of the present invention, is a specific molecular structure or mixture that combines a designated cancer antigen (Ag) to derivatized non-immunosuppressive Tat such that the specific molecular structure triggers an antigen-specific cytotoxic T lymphocyte (CTL) response via the dendritic cell (DC). The cancer vaccine of the present invention can be accomplished via either recombinant DNA or protein constructions or a mixture of Tat* (or immunostimulatory NICE) and antigen. Additionally, immunostimulatory NICE can be used as an adjuvant and mixed with a selected cancer antigen.

The present inventions provides cancer vaccines, adjuvants and immunotherapeutics for preventing and treating cancer, related immunotherapeutics inducing tolerance to organ transplants, to foreign antigens such as Mabs or cytokines used as therapeutics, in autoimmunity, and a method for drug discovery of New Immunomodulatory Chemical Entities (NICE). The present inventions further provide methods for treating cancer and preventing undesirable immune responses to foreign antigens, transplanted organs, and autoimmune diseases with NICE. The present invention further describes motifs and DNA sequences that for the production of small interfering RNAs (siRNA) for the purpose of interfering with AReg development for the treatment of cancer (or Human Immunodeficiency Virus Type 1 (HIV-1) infection), or for interfering with DC development, as a further method to induce antigen-specific tolerance.

Antigen presenting cells (APC), macrophages and dendritic cells are critical in the pathogenesis or response to a variety of diseases, disorders and undesired immune responses. In the present invention, the inventor demonstrates that Tat triggers monocytes to differentiate into antigen-presenting macrophages expressing molecules that specifically suppress the immune response to the presented antigen(s). Treatment for human diseases may introduce foreign protein (biologicals, including but not limited to monoclonal antibodies, insulin, and erythropoietin) or tissues (including organ transplants and stents) where an immune response to the foreign agent is not desired. In autoimmune diseases, certain of the body's own proteins are incorrectly recognized as foreign, resulting in extensive tissue damage. As one preferred example, degradation of collagen type II into peptides can trigger rheumatoid arthritis (RA) in

animals and has been associated with human rheumatoid arthritis. Considerable research has centered on reducing the immune response to these proteins. It is the non-binding theory of the present inventor that the antigen-specific macrophage-induced suppression attributed to Tat can be applied to the reduction of the undesired immune response to certain foreign proteins. Additionally, derivatives of Tat (Tat*), both naturally-occurring and chemically or genetically modified versions, can induce monocytes to differentiate into dendritic cells which can stimulate antigen-specific cytotoxic T lymphocytes important in the beneficial immune response to cancer.

The immunotherapeutics and immunomodulators of the present invention, whether immunostimulatory or immunosuppressive have features in common. In one embodiment of the present invention, Tat or Tat* protein is chemically coupled to an antigen, either a cancer antigen for the cancer vaccine of the present invention or alternatively, a desired tolerogenic antigen for the tolerogen of the present invention. In one embodiment, these conjugates are simply linked using a widely known biotin-avidin system. Biotin, a vitamin, and avidin, a lectin, have a high affinity to one another such that proteins conjugated to biotin bind in a stable manner to proteins conjugated to avidin. Tat (or Tat*) is biotinylated using methods well known to a person of ordinary skill in the art. Similarly, the antigen of interest is conjugated to avidin according to standardized methodology. When biotinylated Tat (or Tat*) and avidin-Ag are combined under concentration and temperature conditions necessary for such a reaction, a Tat-Ag conjugate is formed. The experiments found in Examples 4 and 6 use conjugates constructed in this manner.

Recent surprising discoveries by the present inventor demonstrate that Tat can trigger cytotoxic T lymphocyte (CTL) responses when its dendritic cell (DC) stimulatory activity is isolated away from suppression deriving from alternative antigen-presenting cell (APC) activation. It has not been previously possible to extend the *in vitro* activities of Tat to animals. One obstacle was a failure to understand that the cellular target of Tat activity was a precursor APC as opposed to the T cell, as had been so widely believed by those of ordinary skill in the art. The present inventor determined that Tat stimulates APCs, as opposed to T cells and other cell types, at picomolar concentrations that are physiologic for *in vivo* activity. *In vitro*, APCs are approximately 1000 times more sensitive to Tat than T4 lymphocytes. Due to this discovery, one barrier to the successful use of Tat as an immunotherapeutic, namely achieving concentrations attainable *in vivo*, has been overcome. Thus, Tat has two activities that are the core of the present invention; APC targeting and induction of antigen-specific effects that result from APC activation.

Therefore, an embodiment of the present invention illustrated in Example 2 is that Tat induces monocytes committed to the DC lineage to enlarge into activated, CD86+ DC APCs. The effect of Tat on this population of cells is stimulatory, rather than suppressive because the cells have been previously committed to become DCs. The present inventor has previously demonstrated that chemically derivatized Tat (Tat* or ox-Tat) is immunostimulatory in that it promotes differentiation of monocytes into dendritic cells, which subsequently leads to antigen-specific activation of cytotoxic T lymphocytes. Therefore properly derivatized Tat (Tat*) resulting from chemical or genetic modifications does not induce ARegs from monocyte APC precursors. Tat from

HIV-1 long-term non-progressors (patients infected with HIV-1 who do not progress to Acquired Immunodeficiency Syndrome (AIDS)) and from certain related simian strains of lentivirus are also immunostimulatory rather than immunosuppressive. These natural variations in Tat are important source sequences for the genetically-derivatized Tat* of the present invention.

The present invention presents a model of Tat activation of dendritic cells leading to activation of tumor-specific cytotoxic T lymphocytes. Owing to its monocyte targeting specificity, Tat enters APC precursors, carrying along with it any other protein conjugated to it. At this step the APC is stimulated, so long as Tat is multimeric. Once inside the APC Tat can leave the endosome, the reservoir for almost all soluble proteins, and enter the cytoplasmic space, as indicated through its transactivation of RNA expression. This trafficking property of Tat causes the initiation of major histocompatibility complex (MHC) class I presentation, since association with MHC class I also only occurs in the cytoplasm. The balance and duration of cellular genes that are activated determines whether the APC differentiates into an activated DC that potently presents for CTL activation, or into an AReg that shuts off CTL and other immune responses. Example 3 presents a model of the present invention where the Tat*-Ag conjugate of the present invention is genetically derivatized to favor sustained DC activation and thereby to stimulate a superior CTL response against a cancer, in this case cervical cancer associated with Human Papilloma Virus infection.

The immunosuppressive effects of Tat are mediated by macrophages. When stimulated by Tat, either by natural HIV-1 infection or by Tat uptake, macrophages would induce the Fas Ligand (FasL), which in turn would induce the programmed cell

death (apoptosis) of antigen-reacting, Fas-expressing helper T cells (Example 4). As further described in Example 4, Tat enhances the viability of cultured murine macrophages as long as the macrophages were first activated *in vivo* compared with no prior activation and stimulated with relatively high concentrations of Tat. By comparison, LPS promotes the viability of murine macrophages independently from *in vivo* stimulation, and at the same concentration effective for human macrophages. The non-binding theory of the present inventor to this partial resistance to Tat activity implies a barrier to receptor-mediated Tat uptake in the mouse, which could be partially overcome when macrophage phagocytosis was stimulated by the adjuvant thioglycolate. The same barrier to Tat uptake has prevented therapeutic concentrations from being achieved in humans, but for humans the barrier has resulted from an improper formulation of the Tat drug as monomer so that it cannot bind and activate its APC receptor. The Tat tolerogen of the present invention produces a stable suppression of mouse lymphocyte proliferation and may also serve to suppress an antigen-specific immune response to a variety of protein antigens.

The macrophages responsible for these responses have been identified as antigen presenting cell regulatory macrophages (ARegs). ARegs are also known as "alternatively activated" macrophages (Tzachenis, D. et al. (2002), "Blockade of B7/CD28 in mixed lymphocyte reaction cultures results in the generation of alternatively activated macrophages, which suppress T-cell responses," Blood 99:1465-73). ARegs are stable macrophages expressing the Fas Ligand and secreting the cytokines IL-10 and IL-6 (Novak, N., et al. (2001), "Engagement of FcεRI on human monocytes induces the production of IL-10 and prevents their differentiation in dendritic cells," J. Immunol.

167:797-804; Zhang, H. et al. (1999), "Induction of specific T cell tolerance by Fas Ligand-expressing antigen-presenting cells," J. Immunol. 162:1423-30). AReg are stable and respond in an autocrine and paracrine manner to these two cytokines, as well as in a paracrine manner to IL-4. These cytokines accumulate and switch the immune response from TH1 (based on helper T lymphocytes) to TH2 (based on suppressive T lymphocytes). As these cytokines build up they overwhelm and suppress the immune response and explain why immune responses are normally self-limiting in an antigen-specific manner.

An unexpected observation is that 1,000 fold lower concentrations of Tat (500 pM) trigger this effect on the macrophages, as compared with the concentration required to initiate direct apoptosis of CD4+ T cells (approximately 500 nM). Therefore, at concentration of Tat achievable as an administered immunomodulator, the macrophage effect will preferentially occur over the T cell effect.

The Tat mediated antigen-specific suppression of the present invention is mediated through trans- (intracellular) activation of a CD14+ FasL+ macrophage. Example 5 of the present invention demonstrates that, in human cells, Tat-activated macrophages are immunosuppressive ARegs. At low concentrations of Tat (50 nM), Tat-induced immunosuppression was not only fully reversed by the addition of soluble Fas, but under these conditions, Tat actually became slightly stimulatory (relative to antigen treatment alone). Antibodies to FasL reversed Tat immunosuppression of tetanus responses and enhanced the *Candida* response relative to Tat treatment alone. Suppression could be fully reversed (>95% of control) with the further addition of anti-IL-10 and anti-IL-6 antibodies to the cultures, both cytokines deriving from macrophages

under these culture conditions. The non-binding theory of the present inventor is that a portion of Tat-induced immunosuppression is contributed by induction of FasL, although other Tat-induced factors also could participate in suppressing T cell proliferative responses, especially at higher concentrations of Tat.

When Tat-activated macrophages present more than one antigen, by uptake of soluble antigens, immune responses directed to other antigens would be suppressed as well. This process will blunt T-cell dependent, cellular and humoral immune responses and can be harnessed to induce suppression of these responses in an antigen-specific manner by the administration of Tat-tolerogenic antigen complexes.

An obstacle to the use of Tat as an immunotherapeutic agent has been the reported instability of the molecule and the disparity between Tat's activities *in vitro* and *in vivo*. The unexpected discovery of an activity and a preferred means to deliver mulitmerized Tat (to the APC) provides unique opportunities for drug development based upon increased specific activity. Although Tat is known to stably polymerize *in vitro* and *in vivo*, only the Tat monomer intracytoplasmically trans-activates gene expression (Tosi, G. et al. (2000), "Highly stable oligomerization forms of HIV-1 Tat detected by monoclonal antibodies and requirement of monomeric forms for the transactivating function on the HIV-1 LTR," Eur. J. Immunol. 30:1120-6). Since cytoplasmic trans-activation (as opposed to APC activation) was the only previously defined activity of Tat, it was believed that functional Tat needed to be produced as a monomer. By electrophoretic analysis of a variety of Tat proteins from cell lines, it was determined that biologically active Tat is either a dimer or trimer and stable (Example 1).

In the construction of the immunomodulatory or immunotherapeutic agents of the present invention, multimeric Tat is a preferred formulation.

The present invention has determined that multimeric Tat is necessary for high specific activity. Therefore the sequence, structural and homology features of the Tat protein are critical for the resultant biological activity (Bayer, P. et al. (1995), "Structural studies of HIV-1 Tat protein," J. Mol. Biol. 247:529-35). A further description of the domains of Tat can be found in Example 7. A cysteine-rich motif (aa22-37) of Tat is homologous to other receptor binding ligands. It is therefore modeled that multimeric Tat gains access to the monocyte by binding and triggering a surface receptor. In this regard it has analogy to IFN-γ and TNF-□both of which also activate monocytes as trimeric ligands. It is also known that Tat contains a membrane translocation domain (PTD). After gaining access to the endosome following receptor binding, the PTD permits Tat to freely traffic across the endosomal membrane into the cytoplasm, where it transactivates gene expression, including but not restricted to genes of HIV-1 (Schwarze, S.R. et al. (1999), "In vivo protein transduction: delivery of a biologically active protein into the mouse," Science 285:1569-72). The PTD has been wrongly assumed to facilitate Tat entrance into the cell, which it can only accomplish at high concentrations that have been impossible to attain *in vivo.* The embodiments of this invention will allow for the characterization of the Tat binding motif and lead to the identification of chemical compounds that either tolerize (the tolerogen of the present invention) or trigger CTL responses (the Tat*-Ag conjugate of the present invention).

The Tat of the present invention contains four distinct regions of interest (Kuppuswamy, M. et al. (1989), "Multiple function domains of Tat, the trans-activator of

HIV-1, defined by mutational analysis," Nucleic Acids Res. 17:3551-61) (Example 7). There is a proline rich segment near the amino terminus encompassing amino acids 3-19 (SEQ ID NO. 1). The present inventor surprising determined that this highly conserved region of HIV-1 Tat is a canonical SH3 binding domain. This transduction motif has significant homology to the SH3 binding domain of the mouse gene hairless (*hr*) (SEQ ID NO. 2). Importantly, mice with the *hr* mutation develop an AIDS-like syndrome characterized by poor CTL function, TH1 to TH2 shift, and increased susceptibility to chemical and UV-induced skin cancers. Early evolutionary variants of Tat predominate in lentiviruses that infect monkey species that do not develop immunodeficiency. These variant Tat do not have SH3 binding domains and instead substitute a different sequence again flanked by prolines (SEQ ID NO. 3) into the transduction cassette (SEQ ID NO. 4).

Genetic data indicates this SH3 binding motif regulates monocyte differentiation into ARegs. In Tat proteins which do not contain this SH3 motif or is mutated, monocyte differentiation is directed into DCs which stimulate CTL responses. A second region of interest is a cysteine-rich proposed ligand binding domain (amino acids 22-37, SEQ ID NO. 5) which contains seven conserved cysteines. A third region of interest is the membrane translocation domain (PTD) which encompasses amino acids 47-57. A fourth region of interest encodes a tail

In an embodiment of the present invention, genetic derivatives of Tat, generated through modulating the signal transduction motif defined by the SH3 binding domain, are predicted to drive differentiation predominantly to dendritic cells or immunosuppressive AReg. AReg are also critical contributors to invasion of gastric,

pancreas, and ductal infiltrating breast tumors, as well as components of tolerance in organ transplantation. Proposed variations in the SH3 binding domain that stimulate dendritic cell/CTL responses are replacements of one or both internal residues of the four prolines with neutral amino acids such as alanine or valine. Certain of the SH3 binding cassettes have a third proline internal to the flanking pair, in which case it would be possible to replace any of these three internal prolines. In addition it is a non-binding hypothesis of the present inventor that it is necessary to maintain the two external prolines flanking the SH3 motif/cassette in order to facilitate the proper structure for SH3 binding. This structure with flanking prolines is proposed also to enhance the binding to NICE. In addition, the transduction motif from a non-immunosuppressive human variant Tat, or the motif from the *hr* mutation, can replace amino acids 3-18 of Tat, although the *hr* sequence (SEQ ID NO 2) is predicted to increase suppression. In addition, the stimulatory simian form of Tat (SEQ ID NO. 3), or its human equivalent sequence (SEQ ID NO. 4), can be substituted at this motif. Additional chemical modifications, such as ox-Tat, can be used for stimulation of dendritic/CTL responses and synthetic chemical moieties (NICE) can be constructed to generate an equivalent response.

Variations of Tat for the purpose of inducing tolerance or immune suppression are proposed in where Tat is conjugated to antigen in one of several proposed configurations and further illustrated in Example 7. The nature of the design allows the insertion of any specific antigen into a tolerogen "cassette" described here, in which tolerance will result to that antigen exclusively with an absence of effects on the remainder of the immune system. A particularly beneficial tolerogen construction would be the VH and/or VL regions from any murine monoclonal antibody (Mab), particularly a

Mab directed against a cancer growth antigen. In one embodiment of the present invention, constructs are generated where the tolerogenic antigen is sandwiched between two Tat molecules though linkage at the carboxyl terminus. One of the two Tat molecules is truncated at a point between amino acids 56 and 61. The resultant construct is a dimer with biological activity. In another embodiment of the present invention, constructs inserting the amino acid sequence for the tolerogenic antigen between amino acids 56 and 61. This "insertion" construct would then dimerize to provide a dimeric Tat with divalent antigen characteristics. The cassette could alternatively be trimerized through use of trimerization domains contained in DC stimulatory cytokines such as interferon □or TNF-□Still another embodiment of the present invention is tolerogenic antigen linked to the carboxyl terminus of Tat.

The cassette illustrated for tolerogen construction can be modified to create a cancer vaccine cassette. The cancer vaccine of the present invention substitutes immunostimulatory Tat* for immunosuppressive Tat and replace the tolerogenic antigen with a cancer antigen or portions thereof. In another embodiment of the present invention, cancer antigens can be mixed with NICE or immunostimulatory Tat, which in this example would serve as an adjuvant.

The drug discovery method of the present invention has several distinct steps to differentially screen for NICE active at the SH3 motif. An SH3 mutational library will be constructed that allows the exact identification of high affinity NICE binding suppressive motifs (or dominant negative mutations competing at the same site) but not related, inactive mutations with alternative binding specificities. The mutational library will be constructed firstly on the basis of natural variance in the SH3 motif of HIV-1, some of

which are less active at driving AReg formation, and some of which appear to compete against conventionally suppressive Tat (dominant negative mutations competing at the same site). A second series of mutations will be randomly generated to fill out the library. All sequences will be swapped through polymerase chain reaction (PCR) amplification methods well known in the art into a conventional suppressive Tat (HIV-1 MN), and characterized in the *in vitro* functional biological assay (DC index) developed by the present inventor and described in Example 8. The hybrid Tats demonstrating the highest DC index (most DCs and fewest ARegs) will be additionally evaluated for their activity in competing against native suppressive Tat co-administered to the same APCs. By this strategy, a series of related sequences will be defined having high affinity, reduced affinity, or no apparent affinity for the specific AReg SH3 motif.

NICE will be differentially screened by positive and negative selection against three progressive filters. The first filter for positive binding is conducted at relatively low stringency against the native SH3 binding motif (flanked within prolines). Native SH3 binding motifs that induce ARegs are immobilized on a solid phase support and NICE, purchased in commercial libraries, are allowed to react in varying concentrations for a pre-determined time. Such binding assays are well known to those persons of skill in the art. Appropriate negative controls will be included so that specific binding can be determined and quantitated.

This first positive filter screens a relatively broad number of candidate NICE. The second negative filter reduces and defines candidates by negative binding (flow through) a small series of inactive sequences that "filter out" non-specific NICE, which are trapped by the inactive motifs immobilized on the solid support. In the third and final

filter, NICE will be positively reselected for binding non-identical active motifs, preferentially motifs with very high specific activity identified by their characteristics in the DC index assay. At this third filter, conditions are evaluated such as multimerization and buffering that could enhance high affinity interactions.

NICE scoring positive through the three filter screenings are then evaluated in a functional biological assay (DC index) developed by the present inventor for their ability to induce differentiation of monocytes into either immunostimulatory dendritic cells or immunosuppressive AReg macrophages. This assay is described in detail in Example 8. Appropriate positive and negative controls are included so that increases in the number of DCs or ARegs can be quantitated. In addition, the NICE are tested in a wide variety of doses from 0.1-10^6 mM incubated with the monocytes from 24 hours to one week in order to determine those molecules that have optimal specificity, high specific activity, and favorable toxicity profiles.

Those NICE which are determined to preferentially direct monocyte differentiation into dendritic cells (immunostimulatory NICE) will be evaluated for their ability to induce differentiation of dendritic cells in the hairless mouse model. This animal expresses endogenous SH3 motifs and is immunocompromised. An immunostimulatory NICE of the present invention can overcome the SH3-induced deficiencies in dendritic cells. *Hr* mice will be injected with the NICE, or the NICE will be applied dermally, and subsequently challenged with a cutaneous irritant in delayed-type hypersensitivity assays well known in the art. These cutaneous lesions are first graded visually daily based on size and degree of redness. Skin biopsies are taken from 24 hours to one week after challenge and the number of DCs or Langerhan's cells

determined histocytochemically with monoclonal antibodies specific for mouse dendritic cells or Langerhan's cells. Appropriate positive and negative controls will be included and NICE injected in a wide dose range. The immunostimulatory NICE of the present invention can be administered to mice in this assay though means including, but not limited to intraperitoneal, subcutaneous, intradermal, oral, intranasal, cutaneous and intravenous routes. Those immunostimulatory NICE which perform to criteria in the *hr* mouse model will be considered candidates for immunotherapy of cancer in humans.

In another embodiment of the present invention, immunostimulatory NICE can be used as cancer adjuvants to be administered to patients to boost the immune response to tumor antigens when the construction of a cancer vaccine cassette is not possible or inappropriate. The NICE adjuvant can be administered mixed with the cancer antigen or administered in a different site than the cancer antigen. The cancer vaccine and cancer adjuvants of the present invention can be administered to cancer patients in a variety of doses which will be determined after dose titration studies and methods of administration that will be optimized for the type of cancer.

In yet another embodiment of the present invention, tolerogenic NICE which are determined to preferentially direct monocyte differentiation into ARegs will be evaluated for their ability to induce tolerance after being administered along with the desired tolerogenic antigen. The tolerogens of the present invention will be evaluated for their ability to induce tolerance in normal mice. Mice are injected with tolerogen via a route including but not limited to intraperitoneal, subcutaneous, intradermal, oral, intranasal, cutaneous and intravenous administration. From four hours to one week after receiving a tolerizing agent, the mice are challenged with the corresponding immunogenic antigen

alone. This test assay will be performed with an antigen which is known to induce an immune response in normal mice, such as a human protein. After an appropriate amount of time, 72 hours to 2 weeks, the mice are sacrificed and both T and B lymphocyte responses to the immunogenic antigen are determined using assays well known to those skilled in the art. The immune response in these mice will be validated by challenging the mice with an unrelated antigen which is known to induce an immune response (such as *Candida*) and with antigen that is not expected to induce an immune response (such as a normal mouse protein). Only if the mice react appropriately to these controls will the tolerogen be considered effective. In variations of the above experiment, additional mice will be administered multiple doses of tolerogen before challenging with corresponding immunogenic antigen. It is anticipated that repeated administration of the tolerogen will be necessary to induce and maintain tolerance to certain antigens and this schedule of dosing is optimized for each antigen.

An exciting pharmaceutical method to influence the SH3 control of dendritic cells involves activating RNA interference (RNAi), which results in sequence-specific degradation of the targeted double strand RNA (Fire, A. (1999), "RNA-triggered gene splicing," Trends Genet. 15:358-63, 1999; Zamore, P.D. (2001), "RNA interference: listening to the sound of silence," Nat. Struct. Biol. 8:746-50). Small interfering RNAs (siRNA) are RNA duplexes of 21-23 nucleotides which activate the RNAi pathway through their antisense strand, and silence a gene through targeted degradation of its transcript. siRNAs are being widely developed as an administered drug to suppress selected RNA transcripts. Proposed targets include oncoproteins in cancer and infectious agents. The specificity and sensitivity of the target, an opening on the

transcript free from secondary structure or complexed proteins that allows duplexed siRNA to form, and the actual delivery of the siRNA drug inside the cell are three critical factors governing the outcome of treatment. The sequence of the SH3 binding motif predisposing AReg/DC outcome is an ideal RNAi target. Because its activity occurs within APC, which are actively phagocytic cells, drug delivery is favored. Because the activity occurs at a balance point between stimulation (DC) and suppression (ARegs), small perturbations can be extremely efficacious. Moreover, when acting as an immunostimulant in HIV-1 infection, the siRNA would act dually both as an immunotherapy favoring CTL control through DC formation, and as a direct chemotherapeutic inhibitor of HIV trans-activation by Tat.

An embodiment of the current invention is to create therapeutics for cancer, organ transplantation, and certain infectious diseases using the genetic sequences discovered from analysis of Tat to control DC vs. AReg outcome. Duplexed siRNAs are easily constructed from the sense strand of Tat and Tat variants using methods standard to those skilled in the art. siRNA inhibitory to AReg formation (and thereby useful as immunotherapeutics in cancer) can be constructed from HIV-1 sequences (SEQ ID NO. 6) or from the mouse hairless SH3 binding motif (SEQ ID NO. 7). The protein BLAST search discovering the homology between Tat and the human equivalent of *hairless* is depicted in Example 9. siRNA inhibitory to DC formation, constructed from the human equivalent of the non-suppressive Tat motif (SEQ ID NO. 8), would be suppressive and induce tolerance in organ transplantation and autoimmune disease. HIV-1 infection is an important target for RNAi-based therapeutics, a direction that has been attempted by others and failed to reverse HIV-1 induced immunosuppression

because of the selection of inappropriate sequences within Tat (Lee, M.-T. L. et al. (2003), "Inhibition of Human Immunodeficiency Virus Type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector," J. Virol. 77:11964-72). The present inventor has selected unexpected Tat sequences for RNAi therapeutics which are predicted to overcome the problems previously seen with this treatment modality.

EXAMPLES

Example 1

Biologically Active Tat is Multimeric

Tat protein that is biologically active to stimulate monocytes is multimeric (FIG. 1A-B). In order to determine the size of Tat responsible for activation of antigen-presenting cells (APCs), Tat proteins were isolated and separated by SDS-PAGE electrophoresis. In Panel A, control proteins (lane 1), HIV-1 Tat proteins (lanes 2,3), or recombinant Tat proteins (lane 4) were purified either from CD4+ T cell lines (lane 1), cell lines transduced to express HIV-1 Tat proteins (lanes 2,3), or from *E. coli* transfected with (6xHis) tagged Tat by affinity chromatography to rabbit polyclonal anti-Tat antibodies (lanes 1-3) or His (lane 4). Lanes 2 and 3 contain Tat proteins from cell lines transduced from genetically distinct isolates of HIV-1 that share the property of being synthesized as stable multimers. Protein markers (Mr [kDa]) and location of Tat trimer, dimer and monomer are indicated. Eluted proteins were normalized for OD ^{280}, boiled in buffer containing 1% SDS, and separated under non-denaturing conditions by electrophoresis (PAGE-12%). Proteins from paired gels were either transferred to

nitrocellulose (Panel A) and probed with a monoclonal anti-Tat antibody (Intracel), or (B) electroeluted after each lane was excised into two components, the first > 40 Mr (kDa) ("Upper") and the second <21.5 Mr (kDa) ("Lower"). Human monocytes purified by centrifugal elutriation were cultured in the presence of 50 ng/mL fractionated proteins for three days. Cells were harvested and assayed by flow cytometry (FACS, Becton Dickinson) using an anti-Fas ligand monoclonal antibody (Nok 1, BD Pharmingen) for percent Fas ligand expressing APCs as a measure of activation. Only the fractions containing Tat protein greater than 40 Mr (kDa) contained activity capable of inducing APC activation. The Tat multimer produced by these methods is stable as evidenced by the preservation of the multimer following affinity purification and boiling in SDS.

Example 2

Effects of Tat on the Dendritic Cell Lineage

An additional embodiment of the present invention is that Tat induces monocytes committed to the dendritic cell (DC) lineage to enlarge into activated, CD86+ DC APCs (FIG. **2**). Human monocytes enriched from PBMCs by Percoll density gradient separation and adherance to anti-CD14 coated magnetic beads (Dynabeads M-450, Dynal Biotech) were committed to differentiate into DCs through five days of culture in GM-CSF (100 ng/mL) and IL-4 (100 ng/mL). Committed DCs were cultured overnight either in medium alone (Control), LPS (100 ng/mL), or Tat (50nM), after which they were stained with an anti-CD86 antibody (BD Pharmingen) and analyzed by FACScan for CD86 induction (left panel) or generalized activation (right panel, enlargement into

box R2, shown for Tat-stimulated cells). The MFIs for CD86 expression are 9 (Control), 30 (LPS), and 187 (Tat), CD86 being a specific determinant of DC activation.

Derivitzed Tat reduces AReg differentiation and potently enhances antigen-specific activation of CTLs (FIG. **3**). Tat is chemically derivatized by oxidation (Tat* or ox-Tat) so that it does not induce ARegs from monocyte APC precursors (FIG. **4**). Ten micrograms of Tat/p24 Tat*-Ag conjugate (Ag-Tat*) was administered into the flanks of Balb/C mice in adjuvant on day 0 and day 7. Experimental groups were comparatively immunized in adjuvant with 5 μg of p24 in one flank and 5 μg derivatized Tat in the other flank (Ag & Tat*), or 10 μg of p24 in adjuvant (Ag). Control mice were given two injections of adjuvant. Four mice were treated in each group. At day 14, draining lymph node cells from each animal were harvested and re-stimulated overnight in cultures of irradiated Ap24 (H-2d cells stably transfected to express antigen p24) cells or control non-transfected cells. CTL activity was quantitated as the number of γ-interferon secreting spot forming colonies (SFC)/10^6 plated cells using ELISPOT assays. The background with non-transfected re-stimulators, which was in all cases < 10 SFC/10^6, is subtracted from each point. The results are indicative of three similar experiments.

Example 3

Use of Tat*-Ag Conjugates of the Present Invention for the Treatment of Cancer

In the example illustrated in FIG. **5**, the PrecisionConjugate™ is genetically derivatized to favor sustained DC activation and thereby to stimulate a superior CTL

response against a cancer, in this case cervical cancer associated with Human Papilloma Virus infection.

Example 4

Tat Activation of Macrophages and Suppression of the Immune Response

Recombinant Tat protein is prepared as previously described (Li, C.J. et al. (1995), "Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein," Science 268:429-31) under mildly denaturing conditions and was renatured in the presence of 0.1mM DTT.

Tat activation of monocytes is dose-dependent and saturatable (FIG. **4**). Human monocytes were cultured in increasing concentrations of recombinant Tat for six days at which time they were assayed for Fas Ligand (FasL) induction as a measure of activation by using flow cytometry (FACScan, Becton Dickinson) to quantitate the intensity of staining (mean fluorescence index (MFI)) with an anti-Fas ligand monoclonal antibody (Nok 1, BD Pharmingen). Higher concentrations of Tat did not increase MFI (not shown), and T cells could not be activated with 50 nM Tat (not shown), the plateau stimulatory concentration for APCs.

Tat enhances the viability of cultured murine macrophages as long as the macrophages were first activated in vivo compared with no prior activation and stimulated with relatively high concentrations of Tat (FIG. **6**). APCs were isolated by peritoneal lavage from mice intraperitoneally injected four days earlier with either 2.9% thioglycolate (as adjuvant) or 0.85% saline solution (resting). Harvested washout cells were cultured at 10^6 cells/mL for five days in medium alone (Control, C),

lipopolysaccharide (LPS, 100 ng/mL), or Tat produced as recombinant protein in *E. coli* (Tat, 500 ng/mL). Activation was determined as % enlarged cells (M1 fraction).

The Tat tolerogen of the present invention produces a stable suppression of mouse lymphocyte proliferation (FIG. **7**). Mice were immunized in quadruplicate with a Freund's adjuvant emulsion containing either 5 µg Tat/p24 (recombinant HIV-1 *gag* protein p24) tolerogen (GRP 2) or with 5 µg avidin-p24 (GRP 1) as control. At two weeks residual draining lymph node cells were harvested, pooled within each group, and cultured at 10^5 cells/microtiter well for four days in the presence of graded concentrations of recombinant p24 protein (p24, µg/mL). Proliferation was assayed as a determinant of recall T cell response by quantitating overnight ^3H thymidine uptake (CPM) in a liquid scintillation counter. This response is maintained for up to six weeks.

In addition, the Tat tolerogen of the present invention generates an antigen-specific immune suppression (FIG. **8**). Mice in quadruplicate were immunized at day 0 and boosted at day 7 with an adjuvant emulsion containing either 5 µg Tat/p24 tolerogen (Ag+Tol) or with 5 µg avidin-p24 (Ag Alone) as control. At day 14, draining lymph node cells were harvested and stimulated at 10^5 cells/microtiter culture well either with added antigen (Specific, recombinant p24, 1 µg/mL) or with added anti-T cell receptor monoclonal antibody (NonSpecific, 2C11, 10 µg/mL). Tritiated thymidine uptake (CPM) was determined by liquid scintillation at day 4 of culture. The specific Ag+Tol response is suppressed 98% relative to Ag alone, and is not distinguishable from cells cultured in the absence of stimulants.

Example 5

Tat Suppression is Mediated by ARegs

The Tat mediated antigen-specific suppression of the present invention is mediated through trans- (intracellular) activation of a CD14+ FasL+ macrophage (FIG. **9**). In mice, Tat tolerizes at the T cell level and is maintained for at least six weeks after the initial treatment under the conditions demonstrated in FIG. **7**. A human peripheral blood mononuclear cell (PBMC) population enriched for monocytes by Percoll centrifugation was cultured for four days either in medium containing 5% fetal calf serum (FCS, Control), Tat (50 nM), or LPS (100 ng/mL). Harvested cells were doubly stained with a fluoresceinated (anti-fl1) anti-Fas Ligand monoclonal antibody (Mab), (αFasL-fitc, Nok 1, BD Pharmingen) and with an anti-CD14 rhodamine labeled Mab (αCD14fl2, BD Biosciences, CD14 being a determinant specific to macrophages (MΦ). Cells were analyzed by FACScan (Becton Dickinson) for activation (Forward Scatter), CD14 expression (R2, percent MΦs), and for induction of Fas Ligand (MFI). The T cell population (R1) was CD14- and did not express FasL. Similar results were obtained from cells harvested after 2, 3, 5, or 6 days of culture as for PBMCs harvested at day four.

The present invention demonstrates that, in human cells, Tat-activated macrophages are regulatory and immunosuppressive APC macrophage regulators (ARegs) (FIG. **10**). To define the pathway of Tat immunosuppression, through FasL induction on the macrophage, resulting in loss of helper T cell recall responses, T cell proliferation assays are used with recall antigens, tat and FasL antagonists. FIG. **10**A: Human PBMCs from one individual were cultured in triplicate for 5 days in either

medium (not shown), tetanus antigen (Ag, 0.3 Lf/mL), antigen with the further addition of 50 nM Tat (Ag+Tat) or Ag with 50 nM Tat and recombinant sFas protein (25 μg/mL) to block surface Fas L expressed on macrophages (Ag+Tat+sFas). Tritiated thymidine was added over the last 18 hours, and results are graphed as stimulation index (mean cpm stimulated culture/mean cpm medium control). Results are representative of three similar experiments. At low concentrations of Tat (50 nM), Tat-induced immunosuppression was not only fully reversed by the addition of soluble Fas, but under these conditions, Tat actually became stimulatory (141% relative to antigen treatment alone). FIG. **10**B: Proliferation of PBMCs cultured 6 days with either tetanus or *Candida* antigen alone (Ag), compared with cultures in which Tat (Ag+Tat, 125 nM), or Tat (125 nM) and the antagonistic anti-Fas antibody, ZB4 (250 μg/mL, Upstate Biotechnology) also were added (Ag+Tat+αFas). Results are representative of three similar experiments.

Example 6

Sequence and Homology Features of the Tat Protein

A C-rich motif (aa22-37) of Tat is homologous to receptor binding ligands (FIG. **11**). Additionally, it is known that Tat contains a membrane translocation domain (PTD) (FIG. **12**).

The Tat of the present invention has a proline (P) rich segment near the amino terminus (amino acids 3-19):

Pro Val Asp Pro Asn Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro (SEQ ID NO: 1).

This highly conserved region of HIV-1 Tat is a canonical SH3 binding domain (FIG. **13**).

The mouse hairless (hr) gene also has an SH3 binding motif of amino acids 176-196:

Pro Cys Asp Trp Pro Leu Thr Pro Asp Pro Trp Val Tyr Ser Gly Ser Gln Pro Lys Val Pro (SEQ ID NO: 2).

Homology exists between the human Tat SH3 binding domain (SEQ ID NO.1) and the SH3 binding domain of house hr gene:

3 Pro Val Asp Pro Asn Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro 18 (SEQ ID NO. 1)
Pro Pro Asn Pro Trp Gly Ser Gln Pro
180 Pro Leu Thr Pro Asn----------Pro Trp Val Tyr Ser Gly Ser Gln Pro 193(SEQ ID NO. 2)

Early evolutionary variants of Tat in simian lentiviruses that do not cause immunodeficiency do not have an SH3 binding domain but instead have the following proline-flanked sequence:

Pro Leu Arg Glu Gln Glu Asn Ser Leu Glu Ser Ser Asn Glu Arg Ser Ser Cys Ile Leu Glu Ala Asp Ala Thr Thr Pro (SEQ ID NO. 3)

The human equivalent of the simian sequence above (SEQ ID NO. 3) is:

Ser Asn Glu Arg Ser Ser Cys Glu Leu Glu Val (SEQ ID NO. 4)

Another region of interest is a cysteine-rich proposed ligand binding domain which contains seven cysteines.

Cys Thr Thr Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val Cys (SEQ ID No. 5)

Example 7

Construction of Cancer Vaccine and Tolerogen Cassettes

The schematic structure of native Tat is depicted in FIG. **14**A. There are four distinct domains: (1) Amino acids 3-19 encoding an SH3 binding motif with four proline residues. This region in early simian lentiviruses is much larger, with a conserved proline at position 3 and with homology to acetylation sites; (2) amino acids 22-37 encoding a ligand-binding domain with seven conserved cysteines and (3) amino acids 47-57 encode a membrane translocation domain (PTD). This region varies from 9-11 amino acids, the core nine amino acids being 49-57. Domain four is encoded by amino acids 58-72 consisting of a tail followed by a second exon. Domain can exist in an alternative form 1'.

Cancer vaccine and tolerogen cassettes are constructed in a similar manner. These cassettes have been designed such that multimerization of Tat, which is needed for biological activity according to a non-binding theory of the present invention, is enabled and that antigen, either cancer or tolerogenic, is placed downstream of SH3 and PTD domains. In all four cassettes depicted in FIG **14**B, the NICE identified in the drug discovery method of the present invention can be substituted for domain 1, the native Tat SH3 binding motif. FIG **14**B schematically depicts four possible cassettes for the construction of the immunotherapeutic embodiments of the present invention. Cassette (i) inserts antigen between two Tat molecules, replacing the amino terminus of Tat after amino acid 58. The antigen is linked to the upstream PTD via a glycine-rich linker. In cassette (ii) antigen is linked to the amino terminus of the Tat protein after amino acid 58. In this cassette, the antigen is again linked to the upstream PTD via a glycine-rich linker and downstream of domain 4. Cassette (iii) links antigen to the amino terminus of the complete Tat protein. Cassette (iv) is similar to cassette (ii) except that

immunoglobulin CH region is inserted after antigen. This cassette provides for alternative dimerization since CH regions will spontaneously dimerize. Cassette (v) is the same as cassette (i) except for the absence of domain 4 from the downstream domain 4. Cassette (vi) is the same as cassette (ii) except for the absence of domain 4 downstream from antigen. Cassette (vii) is a variation on cassette (iii) with immunoglobulin CH replaced by either the tumor necrosis factor alpha or the interferon-gamma stem regions which will provide for trimerization of the resultant molecule.

Example 8

In Vitro Bioassay for Monocyte Differentiation

The in vitro ultra-sensitive monocyte Tat bioassay of the present invention is used to assess the immunosuppressant or immunostimulatory activity of the Tat proteins and NICE used in vaccines and tolerogens of the present invention. This assay utilizes fresh monocyte cells substantially purified from human peripheral blood using standard density gradient enrichment procedures or other cell isolation protocols known in the art. The substantially purified monocytes are washed and then cultured in RPMI-1640 supplemented with 10% FBS at 37°C.

The in vitro ultra-sensitive monocyte Tat bioassay is performed using a positive control (FasL, inducing compound) and a negative control (no active compound is added to the culture). Suitable positive controls include, but are not limited to, lipopolysaccharide (LPS) and or tissue necrosing factor (TNF-α) at a final concentration

of 100 ng/mL and 50 ng/mL, respectively. Test samples (Tat preparations) are run at final concentrations from 50 pM to 50nM and include Tat, ox-Tat, NICE and other Tat derivatives and mutants.

The test samples and controls are individually mixed with the substantially pure monocytes seeded at a density of 10^6 cells/mL in round bottom tubes containing RPMI-1640 with 10% FBS (herein referred to collectively as assay cultures). The assay cultures are then incubated for a suitable period of time, preferably from five to six days, at 37°C, in a 5% CO_2 environment.

At the end of the incubation period, cells are removed from each assay culture and the presence of any induced FasL expression (for measurement of differentiation into ARegs) or CD86 expression (for differentiation in dendritic cells) is detected by staining with an anti-FasL or anti-CD86 antibodies and appropriate fluorescent detection agents. After the substantially pure macrophages have been stained, the fluorescence is detected using a fluorescence activated cell sorter (FACS) system. Control staining is performed using the fluorescent detection system alone and subtracted from the specific anti-FasL or anti-CD86 staining seen in the assay cultures. The greater the percentage of FasL positive cells in a given assay culture, the more immunosuppressant the test sample in the assay culture is. Conversely, if the assay culture contains a predominance of CD86 positive cells, the test sample is identified to be immunostimulatory. Negative controls should always remain non-reactive with the antibodies and the positive control should fall within predetermined ranges.

Example 9

siRNAs

Human Tat SH3 siRNA: ccag tagatcctag actagagccc tggaagcatc caggaagtca gcctaa (SEQ ID NO. 6)

Mouse Hairless SH3 siRNA: ccat gtgac tg gcccctgacc ccgcacccct gggtatactc cgggggccag cccaaa gtgccc (SEQ ID NO. 7)

siRNA of the human equivalent of the simian non-immunosuppressive Tat motif: agc aacgagcgga gttcctgcga gctagaggtg (SEQ ID NO. 8)

I claim:

1. A specific molecular structure designated a tolerogen that couples a designated tolerogenic antigen to immunosuppressive lentivirus Tat proteins.
2. The immunosuppressive Tat proteins of claim 1 which have been modified to improve delivery to the AReg.
3. The tolerogen of claim 1 wherein the immunosuppressive Tat protein is replaced by a tolerogenic new immunomodulatory chemical entity (NICE).
4. The tolerogen of claim 1 wherein the tolerogenic antigen is a xenogenic protein.
5. The tolerogen of claim 1 where the tolerogenic antigen is insulin or portions thereof.
6. The tolerogen of claim 1 where the tolerogenic antigen is a monoclonal antibody or portions thereof.
7. The tolerogen of claim 6 where the monoclonal antibody portion is VL or portions thereof.
8. The tolerogen of claim 6 where the monoclonal antibody portion is VH or portions thereof.
9. The tolerogen of claim 1 where the tolerogenic antigen is Factor VIII or portions thereof.
10. The tolerogen of claim 1 where the tolerogenic antigen is conjugated to Tat.
11. The tolerogen of claim 10 where the immunosuppressive Tat protein and tolerogenic antigen are linked via an avidin-biotin linkage system.

12. The tolerogen of claim 10 where the immunosuppressive Tat protein and tolerogenic antigen are linked through genetic engineering of their DNA to provide a recombinant protein.
13. The tolerogen of claim 1 where a fragment of the tolerogenic antigen is conjugated to Tat.
14. The tolerogen of claims claim 1 where the tolerogenic antigen is linked through two Tat molecules.
15. The tolerogen of claim 14 where one of the two Tat molecules is truncated between amino acids 56-61
16. The tolerogen of claim 1 where the tolerogenic antigen is inserted into the Tat molecule between Tat amino acids 56-61.
17. A method for suppressing organ transplant rejection by application of the specific molecular structure of claim 1.
18. The method for suppressing organ transplant rejection according to claim 17 by perfusing the organ with immunosuppressive Tat or tolerogenic NICE prior to transplantation
19. The method for suppressing organ transplant rejection according to claim 17 by implanting a device saturated with immunosuppressive Tat or tolerogenic NICE to allow release of Tat into the transplanted organ.
20. The method for suppressing organ transplant rejection according to claim 19 additionally implanting a device saturated with immunosuppressive Tat or tolerogenic NICE to allow release of immunosuppressive Tat into the transplanted organ.

21. A method for reducing cardiac artery inflammation in response to stent implantation by impregnating stent with immunosuppressive Tat or tolerogenic NICE prior to implantation.
22. A method to reduce ocular inflammation by application of immunosuppressive Tat or tolerogenic NICE-containing eye drops.
23. A method for treating rheumatoid arthritis by administration of the tolerogenic NICE of the present invention.
24. A method for treatment of aberrant immune responses by administration of a mixture of tolerogenic antigen and immunosuppressive Tat in a lipid emulsion.
25. A method for treatment of aberrant immune responses by administration of a mixture of tolerogenic antigen and immunosuppressive Tat in a depot system.
26. A cancer vaccine that couples a tumor antigen to non-immunosuppressive lentivirus Tat protein
27. The non-immunosuppressive Tat protein of claim 26 in which Tat is derivatized to improve targeting and activation of dendritic cells from monocyte precursors.
28. The non-immunosuppressive Tat protein of claim 26 in which Tat is derivatized to improve targeting and activation of Langerhan's cells dendritic cells in the skin.

29. The cancer vaccine of claim 26 wherein the non-immunosuppressive Tat protein is replaced with an immunostimulatory new immunomodulatory chemical entity (NICE).
30. The cancer vaccine of claim 26 where the tumor antigen is an antigen associated with tumor cell growth.
31. The cancer vaccine of claim 30 in which the tumor antigen is selected from a group comprising HPV-E6, HPV-E7, TAG-72, p53, cadherin E, PSA and p53.
32. A method for treating cancer by administrating the cancer vaccine of claim 26.
33. The non-immunosuppressive Tat protein of claim 26 where non-immunosuppressive Tat is derivatized by oxidation of native immunosuppressive Tat.
34. The non-immunosuppressive Tat protein of claim 26 where the amino acids of the Tat SH3 binding domain (SEQ ID NO. 1) are replaced by the transduction motif from a simian Tat (SEQ ID NO. 3) where the lentivirus does not cause immunosuppression in its natural host.
35. The non-immunosuppressive Tat protein of claim 25 where the amino acids of the Tat SH3 binding domain (SEQ ID NO. 1) are replaced by the human equivalent sequence to simian stimulatory Tat (SEQ ID NO. 4).
36. A method for treating cancer by administering an immunostimulatory NICE admixed with tumor antigen.
37. A method for treating cancer by sequentially administering a immunostimulatory NICE and a tumor antigen.

38. A method for treating cancer by pre-treating patients with the tolerogen of claim 1, prior to administration of the cancer vaccine of claim 26.
39. A method for treating cancer by inhibiting AReg formation by administration of a small interfering RNA (siRNA).
40. A method for treating cancer according to claim 39 using the siRNA of SEQ ID NO. 6.
41. A method for treating cancer according to claim 39 using the siRNA of SEQ ID NO. 7.
42. A method for treating cancer according to claim 39 using the siRNA of SEQ ID NO. 7.
43. A method for treating Human Immunodeficiency Virus Type 1 (HIV-1) infection by inhibiting AReg formation by administration of a small interfering RNA (siRNA).
44. A method for HIV-1 infection according to claim 43 using the siRNA of SEQ ID NO. 6.
45. A method for treating HIV-1 infection according to claim 43 using the siRNA of SEQ ID NO. 7.
46. A method for treating HIV-1 infection according to claim 43 using the siRNA of SEQ ID NO. 7.
47. A method for identifying new immunomodulatory chemical entities (NICE) comprising:

a. Reacting a candidate NICE with a Tat SH3 binding domain wherein said Tat SH3 binding domain is bound to a solid phase to identify candidate NICE that bind to said Tat SH3;

b. Identifying said candidate NICE bound to said Tat SH3;

c. Adding said identified candidate NICE to a culture of purified peripheral blood monocytes;

d. Adding Tat having an SH3 binding domain to said peripheral blood monocytes and candidate NICE to form a test culture;

e. Incubating said test culture to allow said monocytes to differentiate into dendritic cells (DC) or macrophages (MPC);

f. Removing said differentiated cells from said test culture and determining the presence or absence of DCs or MPCs.

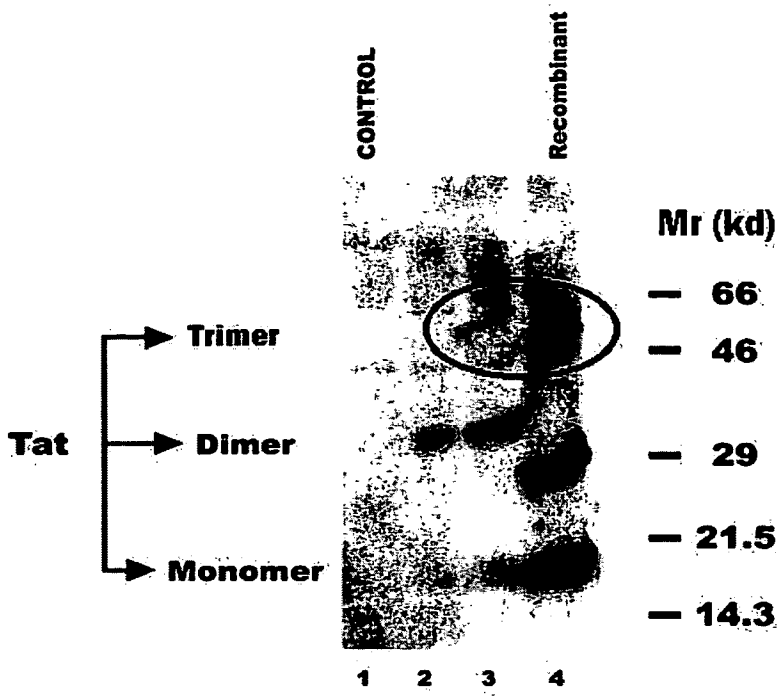
48. The method according to claim 47 further comprising the step of injecting confirmed immunostimulatory NICE from step (f) of claim 47 into an immunosuppressed mouse wherein said immunosuppression results from the presence of an endogenous SH3 binding domain.

49. The method according to claim 48 wherein the said immunosuppressive mouse is a hairless (hr) mouse.

50. A method according to claim 47 further comprising the step of injecting a tolerogenic NICE from step (f) of claim 47 into a mouse and further challenging said mouse with an antigen wherein said tolerance results from the pre-treatment with tolerogenic NICE.

Figure 1

A.



B.

% Activated APCs	Upper	0	18	55	49
	Lower	0	0	3	2

Figure 2

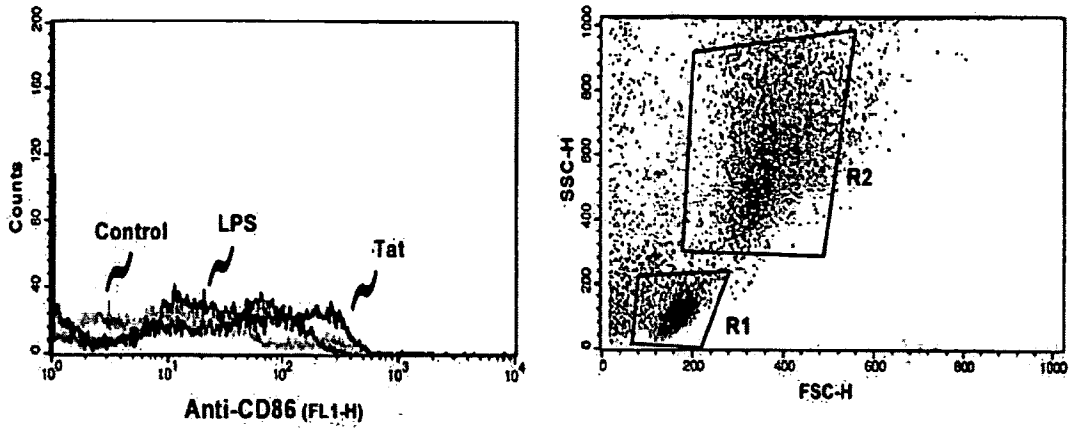


Figure 3

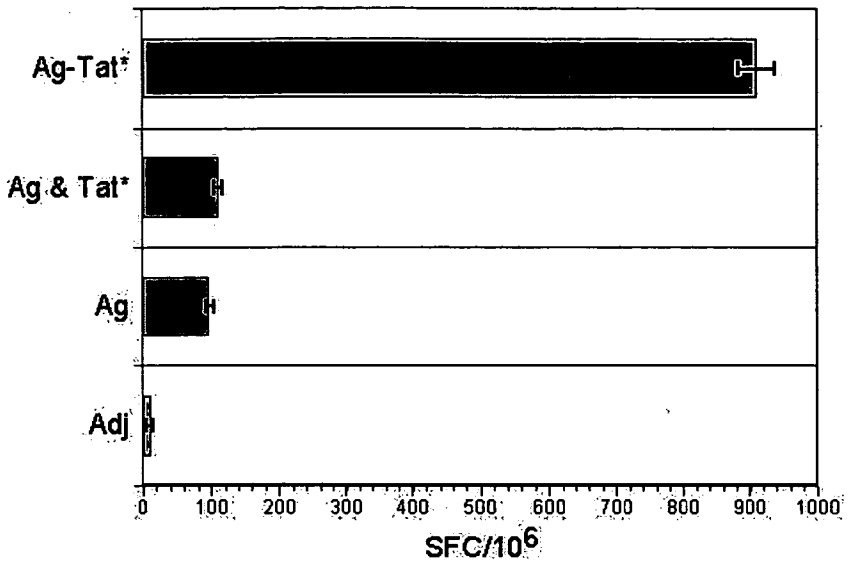

Ag-Tat*
Ag & Tat*
Ag
Adj
0
100
200
300
400
500
600
700
800
900
1000
SFC/10^6

Figure 4

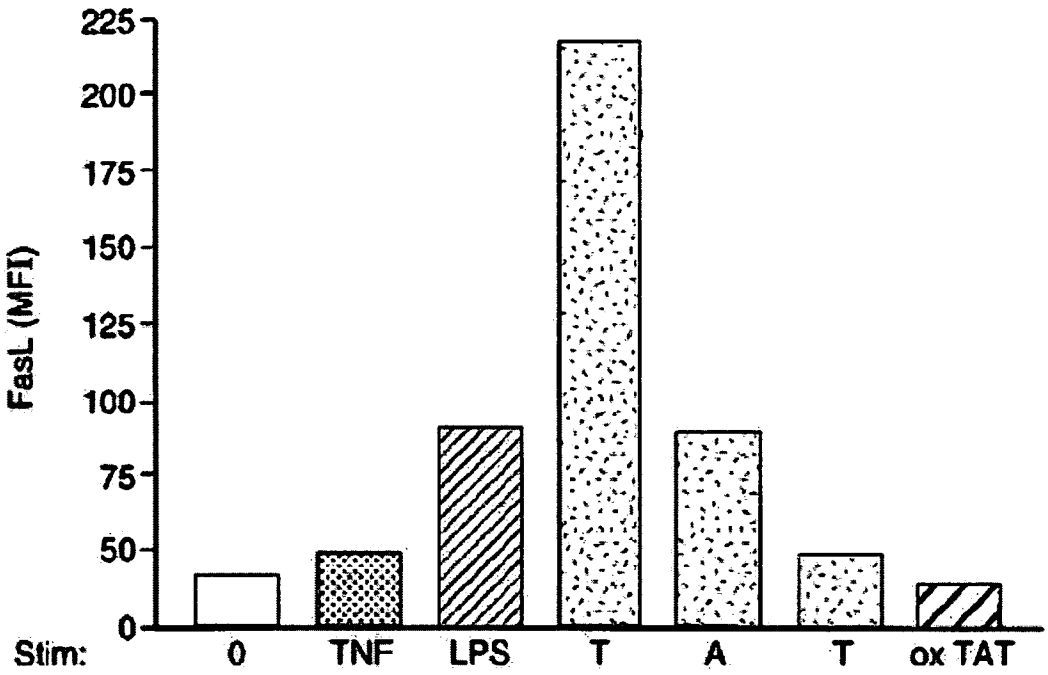
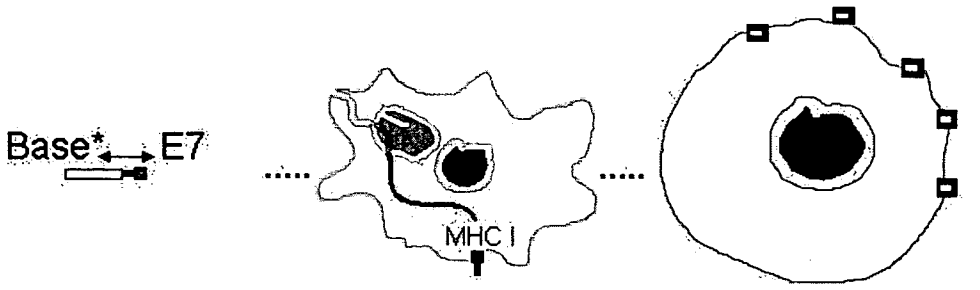


Figure 5



Conjugation to Cancer Ag

Trafficking to DC MHC I

Presentation for CTL Activation

PrecisionConjugates™

Figure 6

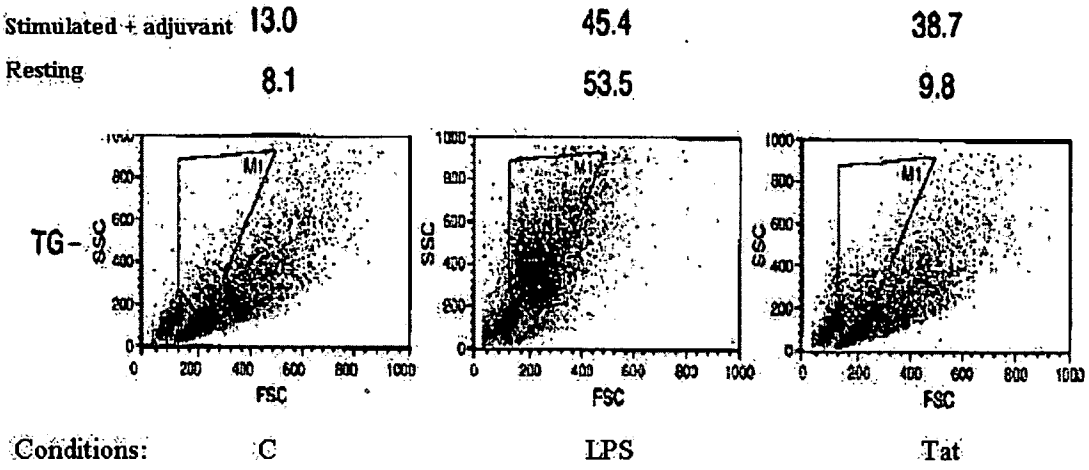

Stimulated + adjuvant
13.0
45.4
38.7
Resting
8.1
53.5
9.8
TG-
SSC
FSC
M1
Conditions:
C
LPS
Tat

Figure 7

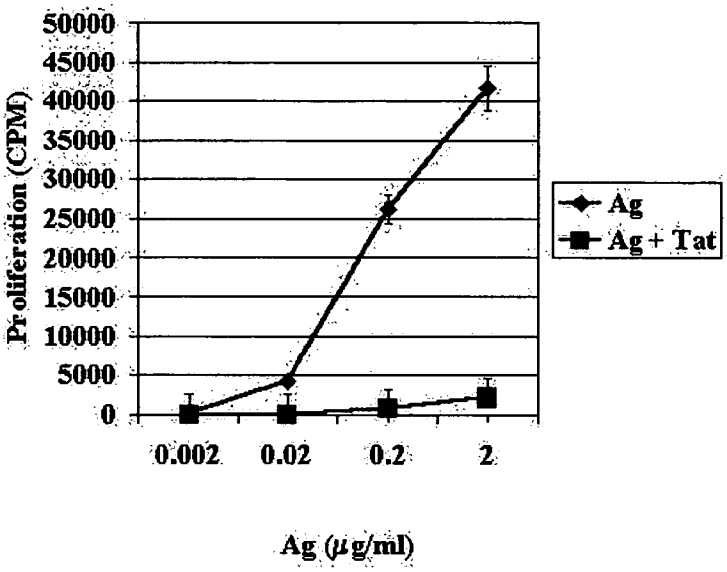

50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Proliferation (CPM)
0.002
0.02
0.2
2
Ag (μg/ml)
Ag
Ag + Tat

Figure 8

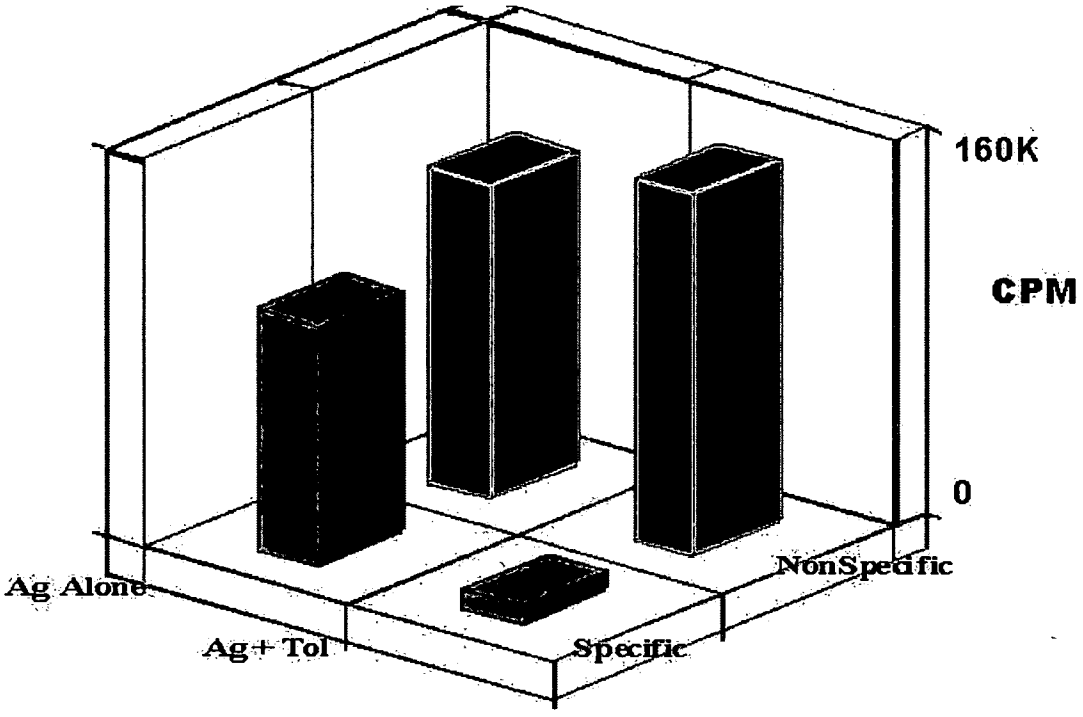


Figure 9

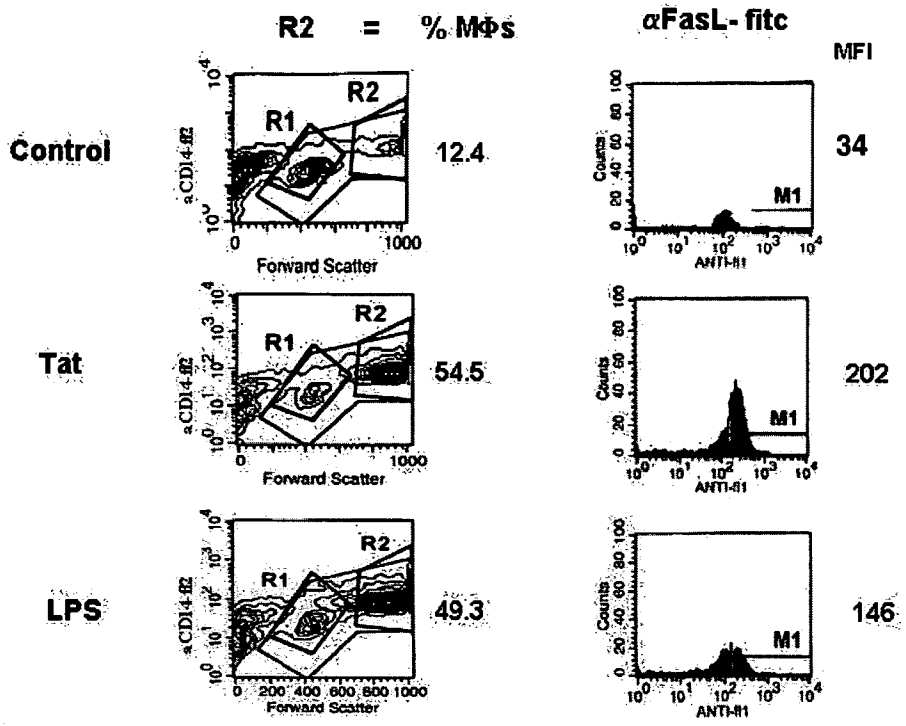

R2 = % MΦs
αFasL- fitc
MFI
Control
Tat
LPS
R1
R2
M1
12.4
54.5
49.3
34
202
146
aCD14-fl2
Forward Scatter
Counts
ANTI-fl1

Figure 10

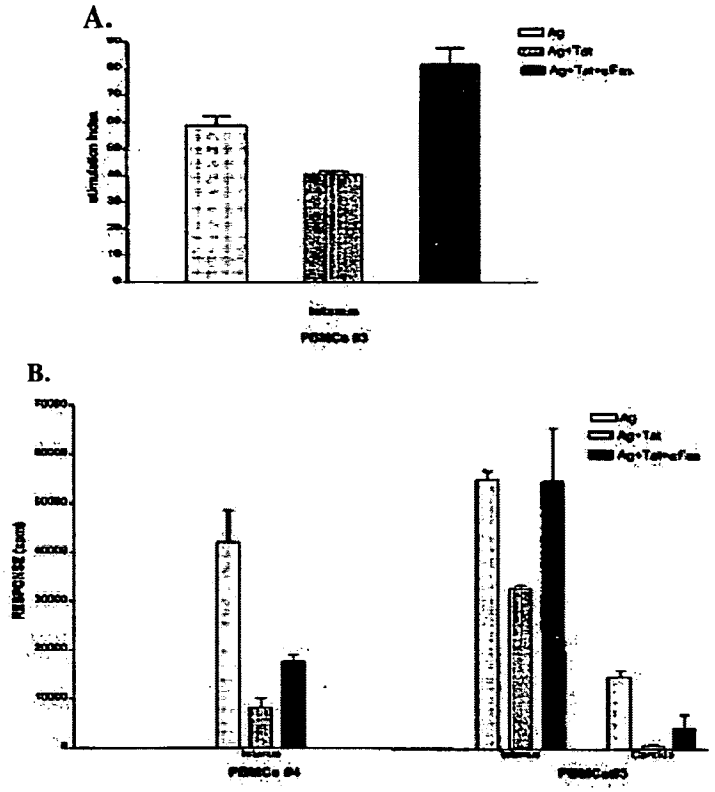

A.
Ag
Ag+Tat
Ag+Tat+qFas
stimulation index
B.
Ag
Ag+Tat
Ag+Tat+qFas

Figure 11

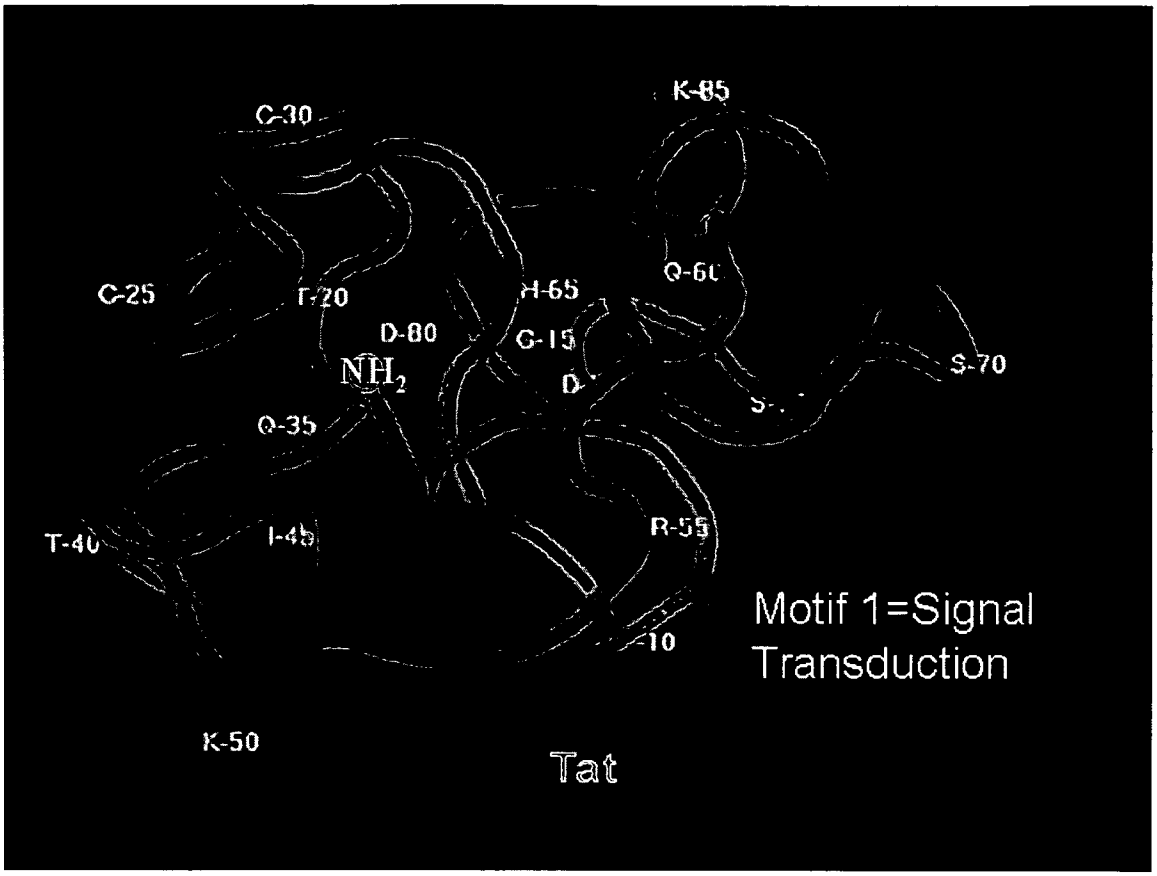


Figure 12

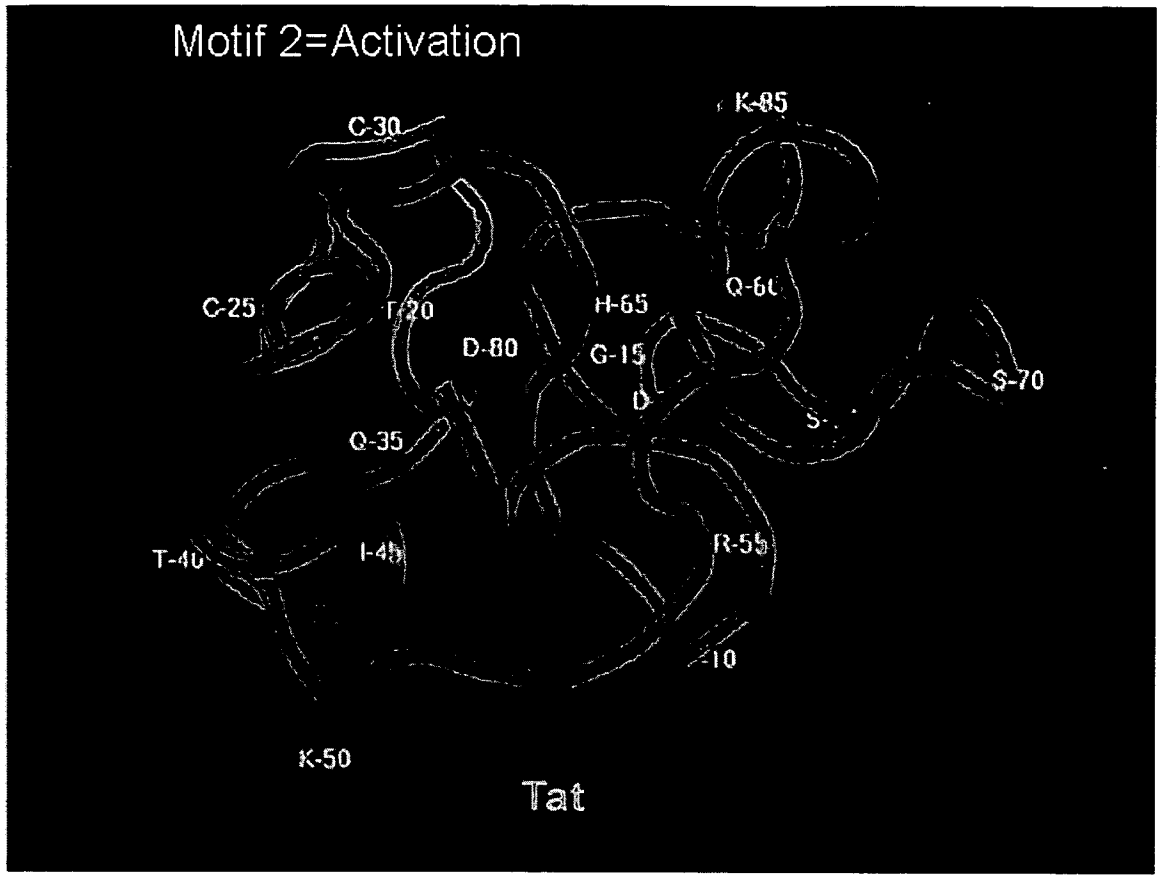


Figure 13

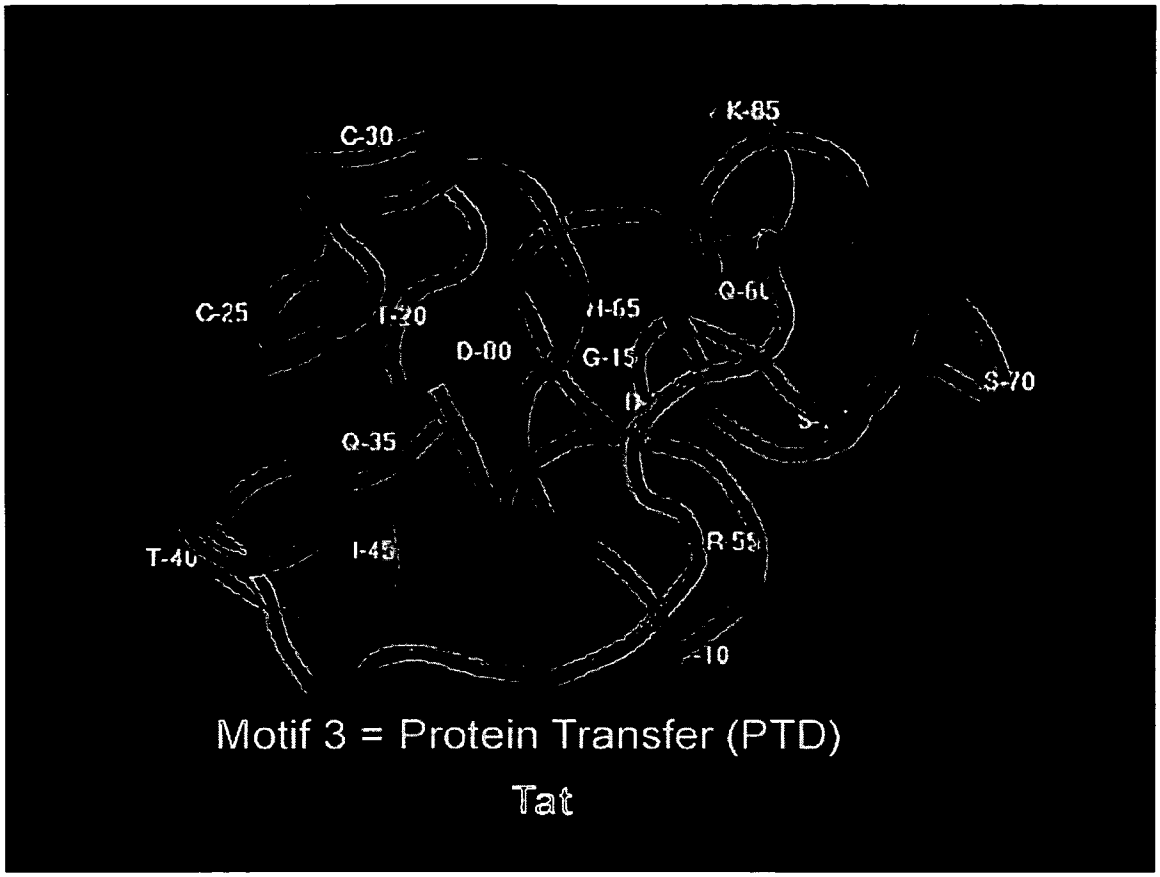
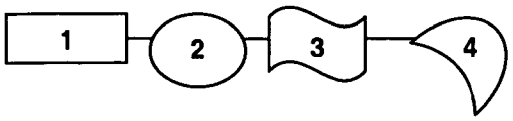
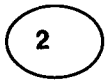


Figure 14

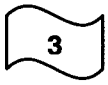
A. Tat



Amino acids 3-18
SH3 binding domain flanked by proline residues with variably 2-3 internal prolines. This proline-flanked region in early simian lentiviruses is much larger, and lacks characteristics for SH3 binding.



Amino acids 22-37
Cysteine-rich ligand binding domain. Typcially contains 7 conserved cysteines.

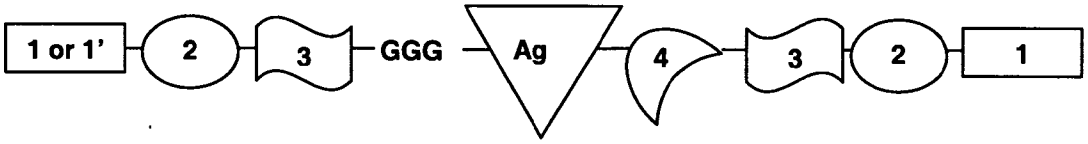


Amino acids 47-57
Membrane translocation domain (PTD). This region varies from 9-11 amino acids, the core nine amino acids are 49-57.

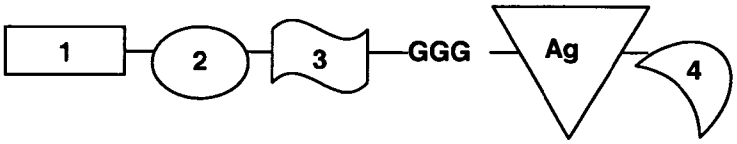
Tail (aa 58-72) and second exon.

B. Anti- Cancer Conjugates and Tolerogen Cassettes

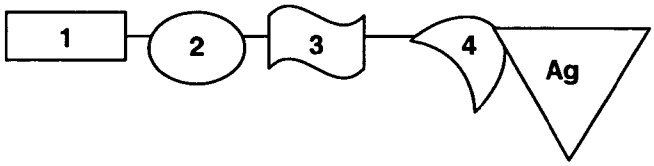
i.



ii.

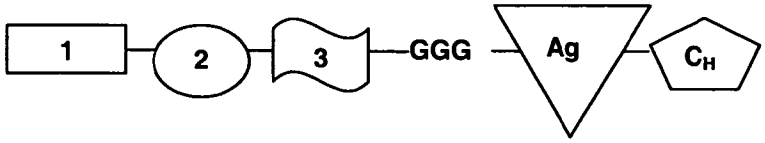


iii.

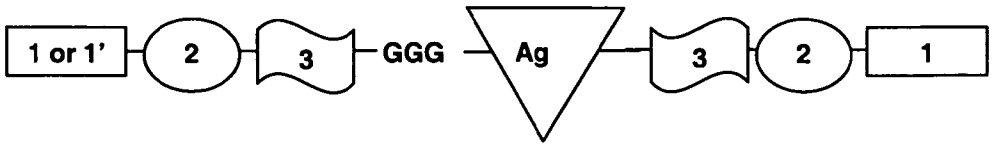


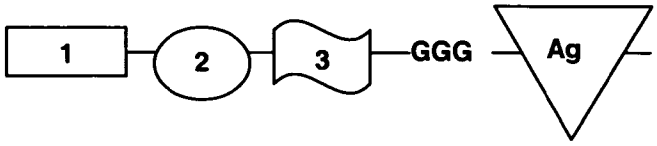
These constructs can also occur with deletions in the tail prior to the second exon.

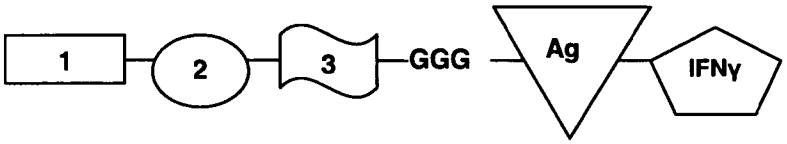
iv.



This construct used for the tolerogen of the present invention inserts the immunoglobulin CH region for dimerization.

v. 

vi. 

vii. 

This construct used for the tolerogen of the present invention inserts the interferon-gamma trimeric stem region for trimerization.